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Characterisation of the Insect Odorant Receptor Complex

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Andrew V. Kralicek

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF DOCTOR OF PHILOSOPHY IN BIOLOGICAL SCIENCE
Abstract

Insect odorant receptors (ORs) are members of a novel family of seven-transmembrane proteins that are thought to form ligand-gated non-selective cation channels. One highly conserved insect OR, Orco, is thought to form the active channel in vivo, with odorant specificity conferred by a panel of ligand-binding OR subunits (OrX). However, little is known about the structural nature of the Orco/OrX complex.

A protein biochemistry approach was applied to directly investigate the oligomeric structure of these receptors without the need for downstream indicators such as changes in fluorescence or electrical current. As a first step to address these questions, a protocol was developed to overexpress, and purify the Drosophila melanogaster (Dm) Orco and six ligand-binding subunits (OrX). Escherichia coli cell-based and cell-free expression systems were not capable of producing these subunits. In contrast, three eukaryotic systems (wheat germ cell-free, baculovirus-mediated Sf9 expression, and HEK-293 cells) expressed each of the subunits, with baculovirus-mediated expression producing the most at 4-6 mg of protein per litre of Sf9 cells.

The receptors were tested for solubility in a panel of 19 detergents, of which Zwittergent 3-16 was the only non-denaturing detergent that could solubilise all OR subunits produced in each expression system. The subunits were purified to homogeneity using Ni-NTA and size exclusion chromatography (SEC) with this detergent. Circular dichroism (CD) analyses demonstrated that the purified subunits were highly structured, having a predominantly α-helical secondary structure. The purified subunits were reconstituted into preformed liposomes using a wheat germ cell-free expression system, and receptors purified from Sf9 cells.

Each of the OR subunits was analysed in a range of detergents, and the presence of higher order structures was determined by Native PAGE. Depending on the OR subunit, monomers, dimers, trimers, tetramers, hexamers, and potentially hexadecamers (16-mers), could be detected. Some oligomers could also be detected by denaturing SDS-PAGE analysis. In contrast, size exclusion chromatography - multi angle light scattering (SEC-MALS) analysis of
the molecular weights of the subunits demonstrated that they were predominantly monomeric with a small proportion forming dimers.

Preliminary surface plasmon resonance (SPR) experiments indicated a potential heteromeric interaction between DmOr22a and DmOrco. Furthermore the DmOrco and DmOr10a subunits were shown to respond to their respective ligands in HEK 293 cells using a calcium sensitive dye assay indicating the potential for these subunits to interact in heterologous cells. However, pull-down assays using His$_{10}$-tagged subunits and Ni-NTA resin did not provide evidence of an interaction between the subunits in any of the expression systems. Similarly incubating the OR subunits with cross-linking reagents did not produce an observable interaction by SDS-PAGE. Single molecule fluorescence photobleaching experiments did not provide any information on the oligomeric structure of these receptor subunits. However, this is likely due to the limitations of the available microscopy equipment.

Finally, each of the six subunits was screened against a panel of 192-480 different crystallisation conditions. This resulted in crystal formation for the DmOrco subunit; however these crystals have not yet been tested for diffraction.

This study is the first to describe the purification of insect ORs and their analysis using a purely biochemical approach. The results obtained provide initial steps towards understanding the structure of the OR subunits and the complexes they from. This research also paves the way for the application of insect ORs in receptor-based biosensors and the generation of novel pest management strategies.
For my wife and kids
Acknowledgements

I would like to start by acknowledging the support and guidance that I have received from my supervisors Richard Newcomb and Andrew Kralicek. Their leadership over the last three years has been a vital component of my PhD research, without which nothing would have been achieved.

I would also like to acknowledge The Plant and Food Research Institute of New Zealand for having the foresight to allow a research associate the opportunity to fulfil his dreams of becoming a PhD scientist. I would never have undertaken this project without their support.

Science should be viewed as a collaborative effort and as such there is always a group of scientists involved in every body of research. My PhD project was no different and I would like to thank the entire Molecular Sensing team at PFR.

Cyril Hamiaux taught me how to purify proteins using nothing but his bare hands and indomitable French spirit (and an AKTA Prime), and also showed me how to create protein crystals. Selene van der Poel was a wealth of information on all things lipid and fluorescent, and whose guidance for the most part kept me on the straight and narrow. Leah Tooman annoyed me at just the right times to aid my procrastination and could always be counted on to listen carefully to a well-constructed rant. Jeremy McCrae could read a chapter in 10 minutes flat and still provide valuable feedback, and was always there with the answer to a stupid Excel question. Bernd, Melissa, Kim, Malcolm, Hyun, John and Ed provided plenty of encouragement and listened to my cries of despair with the requisite amount of compassion and commiseration. Jacob Corcoran taught me that a Frisbee is not just for throwing at your kids but can be used to ruin a good walk, and provide an excuse for an entire days drinking. He also designed and built the HEK 293 functional assay (mad props).

David Christie provided the Flp-In-TREX-Myc-DmOrco HEK 293 cell line and also gave critical feedback on experimental design. Yinqiu Wu and Fiona Clow provided invaluable assistance for the SPR experiments. Dieter Wicher provided the GFP-DmOrco construct.

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Last of all I have to give a huge thanks to my family; a little shout out to my Dad and Mum for bringing me into this world and so on, my brother and sisters, my three kids TadhgEoghan, Caoimhe and Darragh for being the inspiration behind all this, and my beautiful wife for putting up with the long days and longer nights. I love you always.
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<tbody>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>C–terminus</td>
<td>Carboxy–terminus</td>
</tr>
<tr>
<td>DDM</td>
<td>n-Dodecyl β-D-Maltopyranoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
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<tr>
<td>G protein</td>
<td>Guanine nucleotide–binding proteins</td>
</tr>
<tr>
<td>GR</td>
<td>Gustatory receptor</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney 293</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>MALS</td>
<td>Multi angle light scattering</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>N–terminus</td>
<td>Amino–terminus</td>
</tr>
<tr>
<td>OBP</td>
<td>Odorant binding protein</td>
</tr>
<tr>
<td>ODE</td>
<td>Odorant degrading enzyme</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory receptor</td>
</tr>
<tr>
<td>OrX</td>
<td>Ligand-binding olfactory receptor</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neuron</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide electrophoresis</td>
</tr>
<tr>
<td>PBP</td>
<td>Pheromone binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PFO</td>
<td>Perfluoro-octanoate acid</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>Rf</td>
<td>Running front</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT–PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda 9</td>
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List of Publications

The following publication contains the majority of the research outlined in Chapter 2;

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<td>Performed CD experiment and wrote the appropriate method and result section for that experiment</td>
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<td>A.V. Kralicek</td>
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### Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<tr>
<td>A.V. Kralicek</td>
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General Introduction
1.1 Insect Odorant Reception

Olfaction is the key sense for insects and their ability to detect volatile compounds is important for many different activities, from food discovery and location of egg laying sites, to mate recognition and predator avoidance (Benton 2006; Hallem et al. 2006). The odorants that are detected can include small volatile compounds, such as esters and terpenes, and gases such as carbon dioxide. These odorants can be detected at extremely low levels, with moths for example able to respond to the binding of a single sex pheromone molecule, and extremely low levels of plant volatile compounds (Kaissling and Priesner 1970; Angioy et al. 2003).

Olfaction is one of the oldest senses having evolved over 500 million years ago (Vosshall 2000; Robertson et al. 2003), yet there are still many similarities in olfactory systems among highly divergent species (Mombaerts et al. 1996; Kaupp 2010). Odorant receptors (ORs) are membrane-bound proteins expressed in the dendrites of ciliated olfactory sensory neurons, which are embedded in the olfactory epithelium (Buck and Axel 1991). The axons of these neurons project into specific glomeruli, which are housed in homologous structures; the olfactory bulb in mammals and the antennal lobe in insects (Wegener et al. 1993; Hildebrand and Shepherd 1997). Using neuronal input from these structures the brain then builds an odour map that informs response decisions by the animals.

1.1.1 Insect Peripheral Olfactory System

The insect’s antennae are the peripheral olfactory organs that are the equivalent of the nose in mammals (Figure 1-1 a). These antennae are covered in hair-like structures called sensilla which house the olfactory sensory neurons (OSNs), in which the receptors are expressed (Figure 1-1 b). Odorant molecules diffuse through pores in the sensilla and pass into the sensillum lymph where they are likely solubilised by odorant binding proteins (OBPs) or pheromone binding proteins (PBPs), which transport them to the ciliated dendrite of an OSN (Figure 1-1 c). When the odorant binds to the odorant receptor it results in a signalling cascade that causes an increase in intracellular calcium that depolarises the cell and causes the neuron to fire.
There are also other proteins that have been proposed to play a role in insect odorant reception. Benton et al. (2007) demonstrated that Sensory Neuron Membrane Protein 1 (SNMP1) is directly involved in the pheromone response of *Drosophila melanogaster*. SNMP mutants were shown to lack a response to 11-cis-vaccenyl acetate (CVA), while maintaining response to a panel of OR22a-specific ligands. Benton has shown there are hundreds of other insect antennal-specific proteins that have not yet been characterised, any one of which may be involved in odorant perception.

It is important that odorants are inactivated rapidly post detection to enable the odorant sensing system to respond to new stimuli. This is particularly important for insects that rely on chemical cues for navigation while flying, with odorants needing to be inactivated on a millisecond timescale (Ishida and Leal 2005). There is evidence that odorant degrading enzymes (ODEs) can degrade odorants and thus inactivate the signalling mechanism (Ishida and Leal 2005; Ishida and Leal 2008; Durand et al. 2011). The three ODE’s that have been characterised so far are all intracellular antennal esterases shown to degrade pheromones and a single plant volatile ((Z)-3-hexenyl acetate; Durand et al. 2011), but there also must be a more generic molecular mechanism to inactivate signals from other odorants (Leal 2013). OBPs, PBPs and SNMPs have also been proposed to play a role in the deactivation of odors following receptor activation (Vogt et al. 1985; Kaissling 1998; Steinbrecht 1998; Leal 2005; Leal et al. 2005).
1.2 Insect Odorant Receptors are a Novel Family of Membrane Proteins

Insect ORs were predicted to be G protein-coupled receptors (GPCRs) following the discovery of mammalian ORs in 1991 (Buck and Axel 1991). Based on this assumption, a large family of seven transmembrane-domain receptors was found using bioinformatic analyses of the *Drosophila melanogaster* (Dm) genome sequence (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999), and homology probing of an antennal cDNA library (Vosshall et al. 1999). Following this discovery, OR genes were found in many other insect
species (Hill et al. 2002; Krieger et al. 2002; Robertson and Wanner 2006; Bohbot et al. 2007; Wanner et al. 2007; Engsontia et al. 2008; Jordan et al. 2009; Sato and Touhara 2009; Robertson et al. 2010). However, although insect ORs were found to be seven transmembrane domain proteins, they are not phylogenetically related to mammalian ORs (Figure 1-2). Instead they appear to be more closely related to potassium channels (Benton et al. 2006). Furthermore, investigations into the topology of the insect ORs have demonstrated that they are oriented in the membrane with an intracellular N-terminus and an extracellular C-terminus, which is the opposite of conventional GPCRs (Benton et al. 2006; Lundin et al. 2007; Smart et al. 2008; Jordan et al. 2009; Tsitoura et al. 2010).

Figure 1-2: Unrooted neighbor-joining tree of selected protein families. Odorant Receptors (ORs) Class A GPCRs, Methuselah family receptors (Methuselahs), Frizzled receptors (Frizzleds), potassium channels (K channels), and mouse ORs. Sequences were aligned in ClustalX with 1000 bootstrap iterations. (From Benton et al 2006).

Analysis of the relationship between ORs and the gustatory receptors (GRs), from D. melanogaster found that ORs appear to be a greatly expanded lineage derived from the GRs (Robertson et al. 2003; Robertson 2009), with Orco (previously referred to as Or83b (Vosshall and Hansson 2011)) being the most basal of the ORs and therefore the most
closely related OR to the GR superfamily (Figure 1-3). Topology analysis of the GRs indicate that they also have the same inverted topology as the ORs (Zhang et al. 2011). These chemoreceptors are likely a very old lineage of genes, due to the relatively even distribution of the chemosensory receptors in the fly genome, the high divergence levels between ORs and the method of intron acquisition, which is reminiscent of other ancient superfamilies (Robertson et al. 2003).

Figure 1-3: Phylogeny of Drosophila melanogaster ORs and GRs. A neighbour-joining tree of a selection of D. melanogaster odorant receptors (yellow) and gustatory receptors (blue), showing that Orco (red) is the most closely related OR to the GRs.
Unlike vertebrate ORs, which are relatively well conserved with 40%-98% sequence homology (Vosshall et al. 1999), the insect OR family is extremely divergent with 10-75% amino acid homology (Clyne et al. 1999; Gao and Chess 1999; Vosshall and Stocker 2007). Despite this level of divergence, there are more conserved regions that define insect receptors as being members of a common multi-gene family (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Vosshall 2003; Miller and Tu 2008). Miller and Tu (2008) used a hidden Markov model to elucidate highly conserved motifs in Anopheles gamibiae, D. melanogaster and Apis mellifera ORs. They found three conserved motifs in the C-terminal portion of the receptors, an area that covers the last two transmembrane domains and intracellular loop 3. These conserved regions could be involved in ligand binding or protein-protein interactions which could include downstream signalling pathways, or could be a part of the pore-forming region of the ion channel (Clyne et al. 1999; Gao and Chess 1999; Benton et al. 2006; Miller and Tu 2008).
Figure 1-4: Positions of the three highly conserved motifs in *Drosophila melanogaster* Orco. The three conserved C-terminal regions from Miller and Tu (2008) are indicated in yellow.

### 1.2.1 Insect Odorant Receptors are thought to be Ligand-Gated Ion Channels

Insect ORs are members of a novel family of proteins that are not related to GPCRs. As such, there are questions around whether they signal through G proteins or whether other mechanisms are involved. Insect ORs have now been proposed to operate as ligand-gated non-selective cation channels (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008), with the potential to interact with downstream signalling mechanisms (Wicher et al. 2008; Nolte et al. 2013).

#### 1.2.1.1 Role of Orco

Unlike all other insect odorant receptors, Orco is extremely conserved across the class Insecta. It has been found in every insect species that has been investigated to date. The high amino acid identity between Orco orthologues of Diptera (*Drosophila*) and
Lepidoptera (*Heliothes*) would indicate that this OR has an important and well conserved role in insect olfaction (Keller and Vosshall 2003). Knockout experiments in *D. melanogaster*, using both electrophysiological and behavioural techniques, have shown that Orco is essential for proper functioning of the ORs (Larsson et al. 2004). Benton et al. (2006) demonstrated that Orco interacts with conventional ligand binding ORs (OrX) early in the endomembrane system in OSNs, is necessary for correct trafficking of the complex, and is essential to maintain the OR complex within the sensory cilia. Orco is not dependent on other ORs to traffic to the sensory cilia and one of its important functions is to link each of OrX subunits to the transport machinery. Orco orthologues from different species can rescue function in null mutants of *D. melanogaster* (Jones et al. 2005), and the odorant response profile of DmOr35a when expressed with Orco from either *Apis mellifera* or *Ostrinia nubilalis* are very similar (Nichols et al. 2011), indicating a conserved functional role for Orco across the insects.

Orco has been shown to function as a ligand-gated non-selective cation channel when expressed alone, and shows spontaneous activity both *in vivo* and *in vitro* (Larsson et al. 2004; Benton et al. 2006; Nolte et al. 2013). A novel synthetic allosteric agonist for Orco was discovered by Jones et al. (2011) as part of a project to screen mosquito ORs for broadly effective insect repellents. The agonist (VUAA1) was found to potentiate the mosquito OR complex’s response to a natural ligand. Further research found that it elicited concentration-dependent inward currents in HEK 293 cells co-expressing Orco with an OrX, as well as Orco alone, demonstrating that VUAA1 is an Orco agonist and that the currents were Orco dependent (Jones et al. 2011). However, cells expressing a ligand-binding OR (AgOR10) and Orco were more sensitive to VUAA1 than cells expressing just Orco. VUAA1 was subsequently found to elicit similar responses in Orco from *D. melanogaster, Heliothis virescens*, and *Harpegnathos saltator*, demonstrating that it is a broad-spectrum agonist that can activate Orco subunits across multiple insect orders (Jones et al. 2011). Screening a panel of 22 compounds structurally related to VUAA1, Chen et al. (2012) identified two new agonists, as well as a series of antagonists. These agonists and antagonists have a very similar effect on Orco from different insect species, suggesting a conserved binding site. Mutagenesis of conserved aspartic acid residues in TMs five and seven of *D. melanogaster*
Orco (Kumar et al. 2013) suggest an important functional role for this residue at position 466 in TM7. Furthermore mutating the aspartic acid at this position to glutamic acid was demonstrated to increase sensitivity to VUAA1 in comparison to the wild type with both electrophysiology experiments, and calcium influx assays in HEK 293 cells (Kumar et al. 2013).

Thus, the weight of evidence suggests that Orco is required not only to traffic ORs to the OSN membrane, but can act as an ion channel on its own and couples to OrX subunits likely forming a non-selective cation channel.

1.2.1.2 Role of Ligand-binding ORs (OrX)

Initial analyses of the response profiles of D. melanogaster ORs against a panel of different odorants (Hallem and Carlson 2006) suggested that insect ORs range along a continuum from narrowly tuned to broadly tuned, responding to either a single odorant tested or a range of different odorants (Hallem et al. 2004; Hallem and Carlson 2006; Carey et al. 2010; Wang et al. 2010). This led to the hypothesis that insect olfaction was based on a combination of receptors differentially responding to the same ligand (Hallem et al. 2004). The concentration of the compound tested appears to have an effect on the level of response, with high concentrations of benzaldehyde able to activate or inhibit 42% of A. gambiae receptors (Carey et al. 2010). However, recent research raises the possibility that insect ORs are specifically tuned to just one or a small number of compounds, but recognise related compounds at higher concentrations (Bohbot and Dickens 2012; Mathew et al. 2013). Analysis of each of the 21 OrX subunits expressed in D. melanogaster larvae indicated that 19 could be strongly activated by a single odorant out of a panel of 479 tested at low concentrations (Mathew et al. 2013). In the mosquito Aedes aegypti, several ORs have been characterised that are specifically tuned to a single compound while responding to other compounds at much lower levels (Bohbot and Dickens 2009; Bohbot et al. 2011). Within the Drosophila genus Or56a responds exclusively to geosmin, which is a volatile compound produced by some yeast, bacteria and cyanobacteria (Stensmyr et al. 2012). The detection of this compound produces an aversion response in flies, this is proposed to be a mechanism for the fly to avoid toxic mold and bacteria while still detecting edible yeast on
fermenting fruit (Stensmyr et al. 2012). Furthermore, the pheromone receptors of *Bombyx mori* have been shown to elicit a response to a single pheromone molecule (Kaissling 1996), and are highly tuned to the conspecific pheromone (Sakurai et al. 2004; Sakurai et al. 2011).

Although the exact ligand-binding site is not yet known for the OrX subunits, a region between TM3 and extracellular loop 2 in *D. melanogaster* Or85b has been identified as important in the binding of the ligand 2-heptanone, and mutations within TM3 affected ligand sensitivity (Nichols and Luetje 2010). Similarly Nakagawa et al. (2012) found one residue at the border of TM5 and intracellular loop 3 and one within TM6 of *B. mori* that affect ion selectivity in patch clamp experiments. However these sites have not been identified as the exact ligand-binding region in either OrX.

With the discovery that Orco can act as an ion channel when expressed alone in heterologous cells, it has been assumed that the ligand binding ORs must be linked to Orco in some manner to elicit a response to odorants. However, Wetzel et al. (2001) in one of the first heterologous cell expression experiments demonstrated that DmOr43a expressed in HEK 293 cells could elicit electrophysiological responses to odorants without the need for an Orco subunit. Similarly the initial discovery of the pheromone receptor in *B. mori* (BmorOr1) was carried out in *Xenopus laevis* oocytes without an Orco subunit, and responses to the *B. mori* pheromone were detected (Sakurai et al. 2004). In both of these experiments the OrX subunit was expressed with a Gα subunit that was predicted to allow the OrX to interact with endogenous second messenger systems. However, the level of OR response to odorants in heterologous cell systems is greatly increased by co-expression with Orco (Benton et al. 2006; Neuhaus et al. 2005).

### 1.3 OR Signalling Mechanisms

Insect ORs are thought to act as ligand-gated non-selective cation channels. There are however conflicting views on the exact mechanism behind insect olfactory signaling. Sato et al. (2008) described solely ionotropic, G protein independent, currents from ORs expressed in heterologous cell systems using both Ca++ imaging and electrophysiology. They proposed a model in which a ligand-binding OR forms a complex with Orco, producing an odorant-
gated ion channel which has a fast ionotropi
c response. Smart et al. (2008) provide evidence
t that OR signaling is largely independent of G proteins, but that G proteins may be involved
in post-activation modulation. While Wicher et al. (2008) found evidence to support the ion
channel hypothesis as whole cell patch clamp assays demonstrated a rapid odour-induced
activation that decreased rapidly, they proposed an additional signalling route that develops
and decays much slower suggesting that OR ligand binding also produces a metabotropic
response. This metabotropic response is due to the activation of a G protein signalling
cascade which produces cAMP that in turn activates Orco.

If a secondary messenger system is involved in insect olfaction there should be evidence for
a Gα protein interaction. A close homologue of the vertebrate olfactory G-protein Gaolf in
Drosophila (DmGαs) is known to be involved in odorant induced signal transduction (Deng et
al. 2011). In D. melanogaster DmGαs expression can be detected in the third antennal
segment using antibodies against the vertebrate Gaolf protein, and odorant perception is
decreased in mutant flies not expressing DmGαs (Deng et al. 2011). Similarly Talluri et al.
(1995) found a G protein subunit, dGαα-3, which was expressed in D. melanogaster
chemosensory neurons, and when mutated produces flies with reduced responses to
odours (Kain et al. 2008). These responses were reduced further by mutations to the plc21C
gene encoding a phospholipase-C-β. This would indicate that olfactory signal transduction is
mediated at least in part by a phospholipid messenger other than IP3 (Kain et al. 2009;
Hansson et al. 2010).

Early biochemical work on insect olfaction (Breer et al. 1988; Boekhoff et al. 1990; Wegener
et al. 1993; Stengl 1994) found production of inositol 1,4,5-triphosphate (IP3) in antennae
was induced by pheromones, and it was hypothesised that the increase in IP3 may lead to a
rise in intracellular calcium which would control calcium-dependent ion channels. There
was no evidence for the production of cAMP (Boekhoff et al. 1990; Ziegelberger et al.
1990), however production of cGMP was found (Ziegelberger et al. 1990). This evidence was
strengthened by Zufall and Hatt (1991) who found a pheromone gated ion channel (AC1)
that could be activated by both protein kinase C (PKC) and cGMP, but not cAMP or IP3. This
was also the first paper to propose a dual activation system for insect Ors, whereby there is
both a rapid response, and a slower but more sustained response to odorants. However,
studies showing that decreasing the levels of cAMP in ORNs produces abnormal olfactory behaviour would indicate that olfactory signalling relies on the cAMP signalling cascade (Martin et al. 2001; Gomez-Diaz et al. 2004).

Inhibition of phospholipase C (PLC), or PKC, reduces the activation of Orco in response to cAMP in HEK 293 cells, furthermore PKC stimulation activates Orco even in the absence of cAMP (Sargsyan et al. 2011). PKC is known to control function of proteins through phosphorylation (Mellor and Parker 1998), and mutation of the five PKC phosphorylation sites in Orco almost completely eliminated the response to cAMP (Sargsyan et al. 2011). When Orco is expressed alone the ionotropic response can still be measured, however the measured change in cyclic nucleotide concentrations is only evident when a ligand-binding OR is expressed along with Orco. In effect, the OrX subunit may behave like a GPCR interacting with downstream messengers which in turn activate Orco, a cyclic nucleotide-gated (CNG) ion channel (Figure 1-5).

Figure 1-5: Schematic of possible odorant signal transduction mechanisms in insects. The OrX subunit could either interact with the Orco ion channel directly upon ligand binding, or the OR complex could interact with Gα proteins that in turn activate downstream messengers such as the IP₃ or cAMP pathways (indicated by question marks as the mechanisms are currently unknown). Orco can also be phosphorylated by PKC which is itself activated by the increase in intracellular Ca²⁺. Abbreviations: cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; AC, adenylyl cyclase III; PLCβ, phospholipase c beta; IP3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C. Modified from Glatz and Bailey-Hill (2011).
Taken as a body of research there is a large amount of evidence for both ionotropic and metabotropic pathways being involved in insect olfactory signalling. However, there are still questions that remain unanswered, such as: which subunit of the receptor complex activates the second messenger cascades and where are the binding sites between the subunit and the second messenger? Experimental evidence in heterologous cells would point to this being the OrX subunit rather than the well-conserved Orco. There is also the question of whether the Orco and OrX subunits must form a stable complex to enable odorant signalling. Nolte et al. (2013) found no evidence for an Orco-dependent ionotropic mechanism in *Manduca sexta* instead finding that VUAA1 elicited an increase in the background firing rate of the Orco channel, and that in this moth species Orco plays a role of modulating the odorant response threshold and kinetics.

### 1.4 Evidence for a physical interaction between insect OR subunits

There has been little research to date investigating the structure and stoichiometry of the insect odorant receptor complex. The majority of the literature is focussed on de-orphaning and characterising individual OR subunits and elucidating the signalling pathways involved in odorant perception. However a number of reports have provided some evidence for an interaction between the OR subunits, and the formation of a heteromeric complex.

When OrX subunits are expressed alone they are found in the neuronal cell bodies and inner dendritic segment, not the outer dendritic segment where they localise to when co-expressed with Orco (Larsson 2004, Benton et al. 2006). When DmOR43a alone was ectopically expressed in sensory neurons that normally respond only to carbon dioxide, it failed to localise to the dendritic membrane, however when coexpressed with DmOrco it localised to the membrane and induced a response to several known ligands (Benton et al. 2006). Conversely Orco is partially dependent on ORs for stabilization in the membrane but not at all for localisation (Benton et al. 2006). The fact that Orco is necessary for correct localisation of ORs in the dendritic membrane would indicate that they must interact in some structural manner that is stable for at least the duration of the localisation process. OrX subunits have been shown to accumulate on the plasma membrane when expressed in cultured insect cells both with and without the Orco subunit (Smart et al. 2008; Tsitoura et
However, co-localisation in itself does not imply an interaction. Moreover, as these experiments were carried out in insect cells there is also the possibility that the OrX subunits are interacting with an endogenous Orco to enable trafficking to the cell membrane (Smart et al. 2008).

Protein fragment complementation assays (PCA), using yellow fluorescent protein (YFP) as the reporter have demonstrated an interaction between Drosophila OrX and Orco subunits in vivo (Benton et al. 2006). By fusing the N-terminal half of YFP to one protein and the C-terminal half of YFP to another protein, YFP fluorescence will only be observed if the two proteins come into close proximity (within 80 Å of each other). This technique has demonstrated that DmOrco and DmOr43a form multimers, and that DmOr43a is unable to form homodimers without DmOrco present, suggesting that any homo-interaction between ORs is likely to be a result of the formation of a large complex involving Orco.

Resonance energy transfer (RET) experiments in heterologous cells have found evidence for the formation of both homomeric and heteromeric interactions between several ORs including Orco (Neuhaus et al. 2005; German et al. 2013). Bioluminescence resonance energy transfer (BRET) experiments in HEK 293 cells demonstrated that DmOrco can interact with both DmOR43a and DmOR22a, and that both DmOrco and DmOR43a can form homomers. Similarly fluorescence resonance energy transfer in Hi5 cells demonstrated an interaction between DmOrco and DmOR22a and that both these subunits could also form homodimers. However, the stoichiometry of the receptor subunits involved in these interactions is still not known.

A heteromeric interaction between two mosquito ligand binding ORs and Orco was confirmed with co-immunoprecipitation assays using whole-cell lysates of cells transfected with tagged constructs (Tsituoura et al. 2010). Benton et al. (2006) went further to investigate the site of interaction between the OrX subunit and Orco. Chimeric constructs using the N-terminal portion of DmOrco and the C-terminal portion of DmOr43a behave like DmOr43a, as it does not localise to cilia in Orco mutant Drosophila unless it is co-expressed with Orco. This would suggest that the C-terminal portion of ORs is sufficient to bind to Orco and localise to the olfactory cilia. Using a yeast two-hybrid assay they provide evidence for
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the interaction occurring between intracellular loop 3 (IC3) of DmOrco and IC3 of DmOr43a, DmOrco and DmOr22a. However this interaction does not necessarily imply that the subunits are forming a heteromeric ion channel in vivo.

Electrophysiology experiments have demonstrated that the Ligand-binding OrX subunit affects the ion channel in vitro. Nichols et al. (2011) demonstrated that the ligand-binding OrX affects the level of ruthenium red (RR) inhibition observed when expressed with the Orco subunit in X. laevis oocytes, suggesting that the OrX subunits affect the channel pore and thus may be structurally important for pore formation. Pask et al. (2011) went further to examine the ion permeability as well as the RR inhibition of Drosophila OrX/Orco complexes formed with different OrX subunits. When VUAA1 was applied to complexes with different OrX subunits but the same Orco subunit different ion permeabilities were observed, suggesting that the response is due to a heteromeric channel. A similar result was found when the complexes were tested against their respective OrX ligands. By forming an OR complex using the same OrX but Orco from different species they also demonstrated that there were distinct levels of inhibition by RR. Changing the Orco homologue had little or no effect on the ligand binding abilities of the OR complex, providing evidence that Orco is not involved in ligand binding; and thus the OrX subunit is solely responsible for binding odorants; however both subunits could be involved in the heteromeric ion channel (Pask et al. 2011).

The literature to date indicates that there is an interaction between the ligand-binding OrX and the ubiquitous Orco. This interaction is necessary to form a fully functional OR both in vivo and in vitro, and is also necessary to ensure correct localisation of the ligand binding subunit. However, the structure and stoichiometry of the complex remain elusive.

1.5 Potassium ion channels as a model for understanding insect OR structure

In order to formulate hypotheses about the physical interactions between proteins it is useful to have a structural model to work with. Phylogenetic studies on a group of diverse proteins demonstrate that the insect ORs are more closely related to ion channels than
other membrane protein families (Benton et al. 2006), and in particular to Potassium channels, although this relationship is very weak and potentially spurious. However, potassium channels could be used as a basis for elucidating the structure of the insect Ors, until a more appropriate model is found.

Potassium channels are tetrameric membrane proteins that form a membrane spanning pore which allows the movement of K⁺ ions in and out of the cell. All K⁺ channels have at least two transmembrane domains and one pore-loop segment that partially enters the membrane and contains the selectivity filter for K⁺ ions (Choe 2002). They are an ancient lineage of proteins that have been found in every genome sequenced to date. They have also given rise to the cyclic nucleotide-gated channels via the acquisition of a cyclic nucleotide binding domain, and to the Ca⁺ and Na⁺ channels through two gene duplication events (Miller 2000). It is therefore possible that they have also given rise to the insect odorant receptor family.

Like the insect ORs, potassium channels can form both homomeric and heteromeric structures (Telezhkin et al. 2012), however unlike the potassium channels there is no evidence for a tetramerisation domain in the insect ORs. If subunits from the same subfamily of potassium channels are co-expressed they can form functional tetramers, this is true even of subunits from different species (Li et al. 1992). The insect OR, Orco, is known to be functionally active across the species barrier, and can form functional complexes with OrX subunits from different species (Nichols et al. 2011).

Potassium channels have built in mechanisms that regulate the opening and closing of the pore (Choe 2002), however channel function can also be regulated through interactions with other proteins. One example of this is the regulation of the K⁺ channel Kir3 by GPCRs, where the channel interacts directly with the G protein complex (Kubo et al. 1993). There are also K⁺ channels that are inactivated as a result of phosphorylation (Impey et al. 1999; Adams et al. 2000), and by the binding of other protein subunits (Frank An et al. 2000). Sargsyan et al. (2011) have postulated that phosphorylation of Orco is affected by metabotropic pathways and regulates the OR function both in vivo and in HEK 293 cells.
Ion selectivity in potassium channels is regulated by the selectivity filter region which is characterised by the sequence TTVGYGD found in almost all known potassium channels (McCoy and Nimigean 2012). Wicher et al. (2008) postulated that the motif TVVGYLG in the predicted sixth transmembrane helix of Orco is similar to the selectivity filter motif in the pores of K⁺ channels (Doyle et al. 1998). They demonstrated that deletion of two residues to change the sequence TVVGYLG to TVGYG modified the Orco channel properties in whole cell patch clamp experiments in HEK 293 cells, reducing the K⁺ ion permeability with respect to the wild type. Similarly Nakagawa et al. (2012) demonstrated that 13 of the 83 point mutations tested in Orco and Bombyx mori Or1 affect ion selectivity in patch clamping experiments in Xenopus laevis oocytes. Two of these mutations are predicted to be in transmembrane domains (TM) TM5 and TM6 of BmOr1, and a third in TM7 of BmOrco, however none of these mutations affected the potential selectivity domain that Wicher postulated. They could not confirm that these residues were part of a pore forming ion channel, or whether they were merely affecting change in a pore forming elsewhere in the complex or elsewhere in the cell.

As there are many similarities between potassium channels and the insect ORs, it is possible that the insect OR complex might form a functional tetramer. If the insect ORs do form a functional complex then this should be able to be purified intact, or reconstituted from the purified subunits, as has been demonstrated for members of the potassium channel family (Berrier et al. 2004; Asmar-Rovira et al. 2008; Genji et al. 2010; Giudici et al. 2013).

1.6 Aims

The field of insect olfaction is growing at a rapid rate with new discoveries happening on a regular basis. Much is now known about where the receptors are housed, which odorants particular ORs respond to, and where the signals terminate in the brain. However, the structural nature of the receptor complex remains unclear. The majority of research on insect ORs to date has been carried out using a classical genetics-based approach. This has proved to be very useful and the power of the model organism, Drosophila melanogaster, has generated much of the important information for the field particularly in the areas of odorant perception and signal transduction.
The overarching aim of this PhD research is to gain an in-depth understanding of the structural nature of the insect OR complex using a protein biochemistry approach. Each of the protein biochemical techniques are introduced in the introductions of the appropriate chapters. There is very little research into insect olfaction that utilises classical biochemistry techniques. This type of approach could prove extremely beneficial for directly investigating the structural nature of the insect OR complex, without the need for downstream indicators to infer physical associations from insect behaviour, or changes in fluorescence in heterologous assays.

To apply such an approach, the insect OR subunits and the functional complex must be over-expressed and purified. To that end the first aim of this study is to investigate the ability of different expression systems to produce recombinant insect ORs. Two prokaryotic; *Escherichia coli* cells and an *E.coli* cell-free system, and three eukaryotic expression systems; HEK 293 cells, baculovirus-mediated expression in Sf9 cells and a wheat germ cell-free system, will be tested.

The second aim is to develop a protocol for the purification of the ORs and integration into detergent micelles or an artificial membrane to maintain their stability in solution. There are multiple techniques for the production of membrane proteins in a stable soluble environment. However, the expression and solubilisation strategy needs to be optimised for each membrane protein studied. There are currently no protocols available for the purification of insect ORs, therefore I will be taking an empirical approach to identify buffer conditions and detergents needed to obtain pure ORs that can be used in downstream experiments.

The third aim is to investigate the oligomeric structure of the purified OR subunits and the insect OR complex using techniques such as Native polyacrylamide gel electrophoresis (PAGE), Size exclusion chromatography – multi angle light scattering (SEC-MALS) and surface plasmon resonance (SPR).

The fourth aim is to investigate the interaction between the subunits of the OR complex in vitro. Fluorescence resonance energy transfer (FRET), and bioluminescence resonance energy transfer (BRET), experiments have demonstrated that the insect ORs form both
homomers and heteromers in heterologous cells (Neuhaus et al. 2005; German et al. 2013). Pull-down assays and cross-linking reagents will be used to detect if a stable heteromeric ion channel can be formed in vitro. I will also utilise single molecule fluorescence photobleaching to examine the oligomeric structure of the ORs in HEK 293 cell membranes.

The gold standard for determining the structure of a protein or protein complex is to generate a high resolution structure from diffracting crystals. The fifth aim of this project is to begin to investigate the potential to crystallise insect OR subunits for X-ray crystallography studies.
2

Recombinant expression, detergent solubilisation and purification of insect odorant receptor subunits
2.1 Introduction

Insect odorant receptors (ORs) are ligand-gated non-selective cation channels (Sato et al. 2008; Wicher 2010), with some evidence suggesting that their signalling is modulated metabotropically (Wicher 2010). Together with ionotropic glutamate receptors (IRs) (Benton et al. 2009), ORs allow insects to detect volatile compounds associated with mating, predation and food localisation. ORs are localised in the dendritic membrane of olfactory sensory neurons housed in sensilla on the insects’ antennae and maxillary palps (Kaupp 2010). Structurally these receptors contain seven transmembrane helices, but have an inverted orientation in the membrane with respect to G protein-coupled receptors, with an intracellular N-terminus and an extracellular C-terminus (Benton et al. 2006; Lundin et al. 2007; Smart et al. 2008).

The insect odorant receptor complex comprises at least two subunits, one of which is the ubiquitous co-receptor, Orco (Larsson et al. 2004; Vosshall and Hansson 2011), and a second that is a ligand-binding receptor subunit (OrX). Orco is essential for ion channel function in ORs (Larsson et al. 2004), and is highly conserved at the sequence level across insect orders (Krieger et al. 2003). This is in stark contrast with the rapidly evolving ligand-binding ORs (Hansson and Stensmyr 2011; Carraher et al. 2012). Although Orco does not generally respond to odours, a range of compounds that can activate this co-receptor have recently been identified (VUAA1-4; Jones et al. 2011; Pask et al. 2011; Chen and Luetje 2012). Functionally Orco is highly conserved, with Drosophila melanogaster null mutants of Orco capable of being rescued by Orco orthologues from other insect orders (Jones et al. 2005), and in vivo experiments demonstrating that Orco subunits from different species produce similar response profiles to those of odorants when paired with the same OrX (Nakagawa et al. 2005; Nichols et al. 2011). Inhibitor experiments suggest that both Orco and OrX are involved in forming the pore of the ion channel (Nichols et al. 2011); however, this needs to be confirmed. Whilst there is evidence from resonance energy transfer and complementation studies that Orco and OrX form heteromers (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013), the exact size and stoichiometry of the complex or structure of any subunit are unknown. One approach to address these questions is to use
recombinant gene technologies to over-express the OR subunits in either surrogate cells or *in vitro* to facilitate downstream structural and biochemical studies.

Most research on insect ORs has been conducted *in vivo* in *D. melanogaster* (Hallem et al. 2004; Benton et al. 2006; Hallem and Carlson 2006), whilst expression of these novel receptors in surrogate eukaryotic cell systems has been limited. Much of the *in vitro* research has focused on using the cell-based expression systems to study responses of OrXs to various ligands using calcium assays (Kiely et al. 2007; Smart et al. 2008; Benton et al. 2009; Wicher 2010). In addition, some OR subunits have been isolated from stably expressing surrogate cells and used for pull-down assays to investigate receptor subunit interactions (Tsitoura et al. 2010). *D. melanogaster* Orco has also been expressed in a rabbit reticulocyte cell-free system to study the topology of the protein using endogenous and engineered glycosylation sites (Lundin et al. 2007).

### 2.1.1 Aims

In this chapter I aim to investigate the potential of different expression systems to produce recombinant insect OR subunits.

Two prokaryotic (*E. coli* cells and an *E. coli* cell-free system), and two eukaryotic (baculovirus-mediated expression in insect Sf9 cells and a wheat germ cell-free system) will be tested. I also aim to test a diverse panel of detergents for their ability to solubilise OR subunits. I then aim to develop protocols for the purification of His<sub>10</sub>-tagged ORs utilising Ni-NTA and size exclusion chromatography (SEC).

Finally, I aim to reconstitute the OR subunits into pre-formed liposomes either directly using cell-free synthesis, or post-purification using purified material from baculovirus expression.
2.2 Materials and methods

2.2.1 Cloning for Escherichia coli cell-based and cell-free expression studies

Four insect ORs (D. melanogaster DmOr10a, DmOr22a and DmOr43b; Epiphyas postvittana EpOr1) were *de novo* synthesized by Genscript (Piscataway, NJ, USA) with a sequence encoding a 5’ *Not*I site followed by the Tobacco Etch Virus (TEV) protease cleavage site and an *Nde*I site (Kralicek et al. 2011), then an *E. coli* codon-optimised version of the OR gene sequence followed by a 3’ *Bam*HI site. Restriction digests of these four constructs with *Not*I and *Bam*HI enabled cloning into the following derivatives of the tandem λ promoter vector pND707 (Love et al. 1996), pND707-cMBP-his$_6$, pND707-ppiB-his$_6$, pND707-thioredoxin-his$_6$ to produce pND707 derivatives expressing different N-terminally peptide-tagged versions of each OR with a C-terminal his$_6$ tag. Each resulting pND707-NDel-cMBP-Not I-TEV-NDel-OR-BamHI-his$_6$ vector was then digested with *NdEl* to remove the MBP tag, and re-ligated to create pND707 derivatives encoding just the OR-his$_6$ sequence.

In order to create T7 promoter vectors expressing an OR with or without the N-terminal cMBP, PpiB or thioredoxin tags, cloning of the constructs was performed exactly as above except into the following pETMCSI (Miles et al. 1997) derivatives: pETMCSI-cMBP-his$_6$, pETMCSI-ppiB-his$_6$, pETMCSI-thioredoxin-his$_6$, respectively. To create tac promoter vectors expressing non-tagged versions of each OR, the pMALc2x derivative vector, pMALc2-NDel-I-MBP-ProW-Bam HI-his$_6$, was cut with *NdEl* and *Bam*HI, and the ProW gene replaced with each NDel-OR-BamHI fragment, producing four pMAL2c-OR-BamHI-his$_6$ derivative plasmids.

2.2.2 Cloning for insect cell and wheat germ cell-free expression studies

Five odorant receptors from *D. melanogaster* were used in the eukaryotic expression systems, DmOrco, DmOr10a, DmOr22a, DmOr35a and DmOr43b. Each of the odorant receptor genes was tagged with a combination of N-terminal epitope tags to aid in protein purification, and visualisation on western blots.
The tags His\textsubscript{10}-Flag and His\textsubscript{10}-Myc, were added to the N-terminus using PCR amplification. PCR primers were designed to add a TEV cleavage site to the N-terminus of each gene to aid PCR amplification and to enable the subsequent removal of epitope tags. These primers constituted the sequence 5′-GAA AAC CTG TAT TTT CAG GGA (10-15 bp of the target gene)-3′. The methionine-encoding ATG was removed to prevent translation of untagged protein and a new methionine codon added 5′ of the additional tag. These TEV modified constructs were inserted into the pCR8/GW/TOPO vector (Life Technologies, Carlsbad, CA, USA).

Primers were then designed to add a Flag or Myc tag onto the TEV site, and a further round of PCR added the His\textsubscript{10} tag to the N terminus of either the Flag or Myc epitope. This resulted in constructs of the general form Tag-TEV-Gene. All forward primers were designed with a CACC motif at their 5′ end. This allowed the PCR products to be inserted into the pENTR/D-TOPO directional cloning vector (Life Technologies). All primers are listed in Table 0-1. The genes were then gateway cloned into the destination vectors pDEST8 (Life Technologies) for baculovirus expression, and pEU-DEST for wheat germ cell-free expression, using the LR clonase II enzyme (Life Technologies). The vector pEU-DEST was made by digesting pEU-E01-MCS (CellFree Sciences) with EcoRV and KpnI and inserting the PCR amplified attR1-Cm\textsuperscript{r}-ccdB-attR2-KpnI region of pDest8 (Life Technologies). All plasmids were transformed into DH5α chemically competent cells (Life Technologies). Plasmids were Sanger sequenced at Macrogen (Seoul, Korea) using plasmid-specific primers. DNA sequence analysis was performed using GENEIOUS Pro version 6.0.4 (Biomatters Ltd, New Zealand http://www.geneious.com).

Plasmids to be used for cell-free protein expression were transformed into DH5α chemically competent cells (Life Technologies) and DNA was extracted from 100 mL cultures using a Plasmid Midi-Kit (Qiagen). Those being used for wheat germ cell-free expression were further purified by a phenol/chloroform procedure according to the protocol from CellFree Sciences (Yokohama, Japan). The DNA was finally diluted to 1 µg/µL and frozen at -80°C.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Primer function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEV-Orco</td>
<td>5'-GAAAACCTGTATTTTCAGGGAAACCTCGATG-3'</td>
<td>N-terminally tag DmOrco with a TEV site</td>
</tr>
<tr>
<td>TEV-OR22a</td>
<td>5'-GAAAACCTGTATTTTCAGGGATTAAGCAAG-3'</td>
<td>N-terminally tag DmOR22a with a TEV site</td>
</tr>
<tr>
<td>TEV-OR10a</td>
<td>5'-GAAAACCTGTATTTTCAGGGATCCGAGTGGTTA-3'</td>
<td>N-terminally tag DmOR10a with a TEV site</td>
</tr>
<tr>
<td>TEV-OR35a</td>
<td>5'-GAAAACCTGTATTTTCAGGGAGTTCGTTACGTG-3'</td>
<td>N-terminally tag DmOR35a with a TEV site</td>
</tr>
<tr>
<td>TEV-OR43b</td>
<td>5'-GAAACCTGTATTTTCAGGGATTCGGACACTTT-3'</td>
<td>N-terminally tag DmOR43b with a TEV site</td>
</tr>
<tr>
<td>Flag-TEV</td>
<td>5'-CACCATGGATTATAAGGACGATGATGATAAGGAAAACCTGTATTTTC-3'</td>
<td>N-terminally tag with a FLAG epitope</td>
</tr>
<tr>
<td>Myc-TEV</td>
<td>5'-CACCATGGAGCAGAAGCTGATCTCCGAGAGGATCTGGAAAACCTGTATTTTC-3'</td>
<td>N-terminally tag with a Myc epitope</td>
</tr>
<tr>
<td>His-Flag</td>
<td>5'-CACCATGCATCACCATCACCATCACCATCACCATCACCATACATCAGAGCAGAAG-3'</td>
<td>Adds a His tag to a N terminally Flag tagged construct</td>
</tr>
<tr>
<td>His-Myc</td>
<td>5'-CACCATGCATCACCATCACCATCACCATCACCATCACCATACATCAGAGCAGAAGC-3'</td>
<td>Adds a His tag to a N terminally Myc tagged construct</td>
</tr>
</tbody>
</table>
2.2.3 Prokaryotic expression studies

2.2.3.1 *Escherichia coli* cell-based expression studies

For expression trials under the control of tandem λ promoters, the pND707 vectors expressing an OR with or without the N-terminal cMBP, PpiB or thioredoxin tags were transformed into the *E. coli* strain, C43 BL21(DE3) (Miroux and Walker 1996). Five hundred mL cultures were then grown at 30°C with shaking at 200 rpm to either A_{595} of 0.5 or 1.0 in LB containing 50 µg/mL ampicillin. Production of ORs was induced by rapid shift of each culture to 42°C; specifically, the culture was immersed in a 70°C water bath for approximately 2 min. Over-expression was then maintained by placing the cultures at 42°C for a further 3 hours with shaking at 200 rpm.

For expression trials under the control of the T7 promoter, the pETMCSI derivative vectors were also transformed into the C43 BL21(DE3) strain and 500 mL cultures were grown at 37°C to A_{595} of 0.5 in LB containing 50 µg/mL ampicillin. Over-expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the culture was incubated for three hours at 37°C. In the case of the tac promoter expression trials, C43 BL21(DE3) strains transformed with each of the pMALc2x derivative plasmids were grown in 500 mL cultures at 37°C to A_{595} of 0.5 in LB ampicillin. OR synthesis was induced by the addition of 0.4 mM IPTG and the cultures were left at shaking 20°C for a further 40 hours.

2.2.3.2 *Escherichia coli* cell-free protein expression

Continuous exchange cell-free (CECF) protein synthesis of tagged and untagged ORs was performed as described previously (Kralicek et al. 2011; Kralicek 2013) with the following modifications. Three hundred microlitre cell-free reactions were placed in dialysis tubing (12–14 kDa MWCO) and incubated in a screw-capped 15-mL polypropylene tube containing 3 mL of outer buffer. Reactions were then left in a 30°C water-bath shaking at 200 rpm for 16–20 hours. Plasmid templates were included at 16 µg/mL. For protein synthesis using the pND707 derivative templates, 155 µg/mL of *E. coli* RNA polymerase (RNAP) was used to drive RNA synthesis (Guignard et al. 2002). The *E. coli* RNAP was purified according to the protocol described in Burgess and Jendrisak (1975). For protein synthesis using the pETMCSI
derivative templates, 186 µg/mL of T7 RNAP was used. The T7 RNAP was over-expressed and purified following the protocol of Ozawa et al. (2004), with the modifications described in Xun et al. (2009).

2.2.4 Eukaryotic expression studies

2.2.4.1 Baculovirus-mediated protein expression

The pDest8 constructs were transformed into DH10Bac cells (Life Technologies), with transformants selected on LB agar plates supplemented with the requisite antibiotics at 100 µg/mL, and incubated overnight at 37°C. Individual colonies were screened by PCR to confirm the presence of an insert of the correct size using M13 primers. Colonies with the correct insert were grown in 4 mL of LB media supplemented with the requisite antibiotic and incubated at 37°C overnight with shaking at 200 rpm. Bacmid DNA was isolated following the protocol provided by Life Technologies and were chemically transfected into Sf9 cells using Escort IV (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s instructions, and statically incubated at 28°C for 72 hours. The supernatant was decanted and centrifuged at low speed to remove any cells and debris, 500 µL was used as a stock to infect 50 mL of Sf9 cells at ~2 x 10⁶ mL⁻¹. These shaker cultures were incubated for 48 hours at 28°C on an orbital platform rotating at 100 rpm. The cells were then pelleted and the supernatant removed, and foetal bovine serum (Life Technologies) was added to a final concentration of 2%. This stock was titred using an antibody assay (Makela et al. 2010) and used to infect cells for subsequent experiments.

The Sf9 cells were maintained in a shaking solution of SF900II media (Life Technologies) at 27°C and 100 rpm. For baculovirus-mediated expression experiments, the cells were diluted to 2 x 10⁶ mL⁻¹ and inoculated with virus at a known multiplicity of infection (MOI). Optimal MOI was deduced by comparing expression levels on a western blot from a range of MOIs between 0.2 and 6. The optimal MOI varied across viral stocks and constructs (data not shown). A harvesting time of 72 hours post infection was identified by comparing expression levels on a western blot at four time intervals, 24 hours, 48 hours, 72 hours and 96 hours post infection (data not shown).
2.2.4.2 Wheat germ cell-free protein expression

The WEPRO7240H kit from Cell Free Sciences (Yokohama, Japan) was used for eukaryotic cell-free expression. This kit is designed for the expression of his-tagged proteins, as the WEPRO extract has been stripped of endogenous proteins that bind to his-affinity resins. Wheat germ cell-free expression requires transcription and translation to be carried out separately. Transcription reactions were set up in 1.5 mL microcentrifuge tubes with a total volume of 20 µL as per the manufacturer’s instructions and incubated at 37°C for 6 hours. Translation reactions were set up in individual wells of a 96-well microtitre plate using 10 µL of the transcription reaction mixed with 10 µL of the WEPRO mix and 0.8 µL of creatine kinase then overlaid with 206 µL of feeding solution as per the manufacturer’s instructions. The reaction was incubated at 15°C for approximately 20 hours.

2.2.5 SDS-PAGE and Western blot analysis of OR expression

All expression experiments were assessed on 4-12% SDS-PAGE gels (Life Technologies) with Coomassie staining. Samples were placed in a loading solution containing 3M urea and left at 37°C for 30 min to ensure denaturation. Western blotting analysis was carried out by transferring the proteins onto a nitrocellulose membrane using an iBlot (Life Technologies), then blocked with 5% non-fat milk powder in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween® 20, pH 7.5), and incubated with the relevant primary antibodies (anti-MBP, (New England BioLabs), anti-polyhistidine, (Sigma-Aldrich), c-Myc (Santa Cruz Biotech, USA) and anti-flag M2 (Sigma-Aldrich)) for 1 hour each. Epitope-containing bands were visualised using an alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich) with 5 mL of NBT/BCIP solution (Pierce, USA).

2.2.6 Detergent solubilisation trials of over-expressed ORs

Nineteen detergents (Table 0-2) were assessed for their ability to solubilise insect OR subunits expressed in either the baculovirus or wheat germ cell-free expression systems. Each detergent was made up as a 10% stock w/v in water and stored at 4°C.

For the detergent trial where solubilisation of baculovirus-produced protein was studied, Sf9 cells were grown at 27°C on a shaking platform at 100 rpm. Cells were diluted to 2 x 10^6
ml\(^{-1}\) in 90 mL of Sf900II media (Life Technologies), and inoculated with virus at a MOI ranging from 0.2-1 (this varied for each construct to produce optimum levels of expression). Infected cells were incubated for 72 hours at 27\(^{\circ}\)C and 100 rpm shaking, and the cell pellet was collected by centrifugation at 3800g for 10 min at room temperature. The pellet was resuspended in buffer (50 mM NaH\(_2\)PO\(_4\), pH 7.5, 150 mM NaCl, 1x protease inhibitor cocktail (Roche, Basel, Switzerland), 25 U/mL Benzonase (Sigma-Aldrich, Milwaukee WI)) at 1 mL buffer for every 1.8 x 10\(^{7}\) cells, then lysed by two passes through an Emulsiflex C5 emulsifier (Avestin, Germany) at 10,000-15,000 psi. The sample was centrifuged at 1000g for 5 min to remove whole cells and nuclei. The supernatant was removed and 225 \(\mu\)L aliquots were placed in 1.5-mL microcentrifuge tubes with 25 \(\mu\)L of 10\% w/v detergent stock added to each tube to give a final concentration of 1\% w/v. The samples were rotated at room temperature for 1 hour or 24 hours at 10 rpm and 200 \(\mu\)L of each sample was centrifuged in an Airfuge (Beckman Coulter, USA) at 30 psi (\(\sim\) 160,000 g) for 30 min. The supernatant was removed and 10 \(\mu\)L was loaded on a 4-12% SDS-PAGE gel (Life Technologies). The pellet was resuspended in 200 \(\mu\)L of 2\% SDS, 4 M urea, 100 mM DTT and incubated at 37\(^{\circ}\)C for 30 minutes, 10\(\mu\)L of this solution was loaded on the gel as the unsolubilised fraction.

For detergent trials where solubilisation of wheat germ cell-free expressed protein was studied, six reactions were pooled and 49.5 \(\mu\)L aliquots were mixed with 5.5 \(\mu\)L of a 10\% w/v stock of each detergent. The samples were mixed on a rotator for 1 hour at 10 rpm at room temperature, and then spun in an airfuge (Beckman-Coulter, USA) at 30 psi (\(\sim\)160,000 g) for 30 min. The supernatant was then removed and 10 \(\mu\)L was loaded on a 4-12% SDS-PAGE gel (Life Technologies, USA). The pellet was resuspended in 50 \(\mu\)L of 2\% SDS, 4 M urea, 100 mM DTT and incubated at 37\(^{\circ}\)C for 30 minutes, 10 \(\mu\)L of this solution was loaded on the gel as the unsolubilised fraction. Western blotting analysis was carried out as previously described.
Table 0-2: Detergents that were screened for their ability to solubilise insect odorant receptors.

<table>
<thead>
<tr>
<th>Class</th>
<th>Detergent</th>
<th>Supplier (catalogue number)</th>
<th>CMC (mM)</th>
<th>CMC %</th>
<th>Average micellar weight (kDa)</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Ionic</td>
<td>Brij 35</td>
<td>Sigma-Aldrich (P1254)</td>
<td>0.09</td>
<td>0.01</td>
<td>48000</td>
<td>(Ishihara 2005, Klammt 2005)</td>
</tr>
<tr>
<td>Non-Ionic</td>
<td>Brij 58</td>
<td>Sigma-Aldrich (P5884)</td>
<td>0.08</td>
<td>0.01</td>
<td>79000</td>
<td>(Berrier 2004, Lian 2009)</td>
</tr>
<tr>
<td>Non-Ionic</td>
<td>Brij 78</td>
<td>Sigma-Aldrich (P4019)</td>
<td>0.05</td>
<td>0.01</td>
<td>unknown</td>
<td>(Luche 2003)</td>
</tr>
<tr>
<td>Non-Ionic</td>
<td>Brij 98</td>
<td>Sigma-Aldrich (P5641)</td>
<td>0.03</td>
<td>0.03</td>
<td>unknown</td>
<td>(Uematsu 2012)</td>
</tr>
<tr>
<td>Non-Ionic</td>
<td>Dodecyl maltopyranoside (DDM)</td>
<td>Affymetrix (D310)</td>
<td>0.15</td>
<td>0.08</td>
<td>50000</td>
<td>(Berrier 2004, Klammt 2005, Du 2010)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Digitonin</td>
<td>Sigma-Aldrich (D1407)</td>
<td>&lt;0.5</td>
<td>0.08</td>
<td>70000</td>
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<tr>
<td>Anionic</td>
<td>Triton X-114</td>
<td>Sigma-Aldrich (X114)</td>
<td>0.20</td>
<td>0.01</td>
<td>unknown</td>
<td>(Musunuri 2012, Zaini 2013)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Triton X-100</td>
<td>BDH (30632)</td>
<td>0.23</td>
<td>0.02</td>
<td>80000</td>
<td>(Klammt 2005, Liang 2011)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Saponin</td>
<td>Sigma-Aldrich (47036)</td>
<td>0.01-0.1</td>
<td>0.001-0.01</td>
<td>unknown</td>
<td>(Schuck 2003)</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>Octyl β-D-glucopyranoside</td>
<td>Carbosynth (DO05161)</td>
<td>19</td>
<td>0.73</td>
<td>25000</td>
<td>(Klammt 2005)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Tween 20</td>
<td>BDH (663684B)</td>
<td>0.06</td>
<td>0.01</td>
<td>unknown</td>
<td>(Berrier 2004, Klammt 2005)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Sodium Cholate</td>
<td>Sigma-Aldrich (C1254)</td>
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<td>0.60</td>
<td>900-1300</td>
<td>(Liang 2011)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Sodium deoxycholate</td>
<td>Sigma-Aldrich (D6750)</td>
<td>2-6</td>
<td>0.083-0.249</td>
<td>1200-5000</td>
<td>(Zhou 2006)</td>
</tr>
<tr>
<td>Anionic</td>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>BDH (442444H)</td>
<td>8.3</td>
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<td>18000</td>
<td>(Klammt 2005)</td>
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<td>Anionic</td>
<td>N-Lauryl sarcosine</td>
<td>Sigma-Aldrich (L5777)</td>
<td>14.6</td>
<td>0.43</td>
<td>600</td>
<td>(Li 2011)</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>Lauryldimethylamine-N-Oxide (LD)</td>
<td>Affymetrix (D360)</td>
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<td>0.02</td>
<td>17,000</td>
<td>(Columbus 2006)</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>Zwittergent 3-14</td>
<td>Calbiochem - Merck (693017)</td>
<td>0.1-0.4</td>
<td>0.01</td>
<td>30,000</td>
<td>(Briskin 1984, Kokeguchi 1994)</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>Zwittergent 3-16</td>
<td>Calbiochem - Merck (693023)</td>
<td>0.01-0.06</td>
<td>0.003-0.018</td>
<td>60,000</td>
<td>(Mills 1983, Squire 1984)</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>CHAPS</td>
<td>Sigma-Aldrich (C5070)</td>
<td>2.4-10</td>
<td>0.49</td>
<td>6,000</td>
<td>(Berrier 2004, Klammt 2005)</td>
</tr>
</tbody>
</table>
2.2.7 Purification of recombinantly expressed insect OR subunits

The purification of recombinantly expressed insect OR proteins required the preparation of membrane material by differential centrifugation followed by affinity purification using the N-terminal deca-histidine tag, followed by a final gel filtration step.

To his-tag affinity purify protein from baculovirus-infected Sf9 cells, 180 mL at 2 x 10^6 mL^-1 were infected with baculovirus at an MOI of 1, and incubated at 27°C for 72 hours. The cell pellet was collected by centrifugation at 3800g for 10 min at room temperature and then resuspended in 20 mL of resuspension buffer A (50 mM NaH₂PO₄ pH 7.5, 150 mM NaCl, 1x protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)), with 25 U/mL Benzonase, then lysed by two passes on an Emulsiflex C5 emulsifier (Avestin, Germany) at 10,000-15,000 psi. The sample was then centrifuged at 1000g for 5 min to remove whole cells and nuclei. The supernatant was removed and spun at 100,000g for 1 hour at 4°C. The membrane pellet was resuspended in 6 mL of buffer A (50 mM NaH₂PO₄ pH 7.5, 150 mM NaCl, 1 x protease inhibitor cocktail (Roche, Germany)), with 10 mM MgCl₂ and 1% w/v detergent (Zwittergent 3-16) and rotated for 1 hour at room temperature at 10 rpm. The sample was then centrifuged at 100,000g for 1 hour at 4°C. The supernatant was removed and loaded onto a 1 mL Ni-NTA column (GE Healthcare). The column was washed in ten column volumes of buffer B (50 mM NaH₂PO₄ pH 7.5, 0.2 mM Zwittergent 3-16) with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes buffer B with 150 mM NaCl and 250 mM imidazole. Purity was assessed on Coomassie stained SDS-PAGE gels and western blotting as described above.

To his-tag affinity purify protein from wheat germ cell-free reactions, 3 (DmOrco) or 12 (DmOr22a) standard (20 µL) reactions were pooled and spun in an airfuge for 1 hour at 30 psi (160,000g). The resulting pellet was resuspended in 3.5 mL of buffer C (50 mM NaH₂PO₄ pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1% Zwittergent 3-16) and rotated for 1 hour at room temperature at 10 rpm. The sample was centrifuged at 100,000 g for 1 hour at 4°C. The supernatant was removed and loaded onto a 1 mL Ni-NTA column (GE Healthcare). The column was washed in 10 column volumes of buffer B with 300 mM NaCl and 50 mM
imidazole, and a further 10 column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes of buffer B with 150 mM NaCl and 250 mM imidazole. Purity was assessed as described above.

Purification for both preparations was completed with a final size exclusion chromatography (SEC) step. The first two elution fractions from the respective Ni-NTA purifications were pooled and centrifuged at 20,000g for 5 min to remove aggregates and contaminants. Then 300 µL of the supernatant was injected onto a Superose 12/200 GL column (GE Healthcare) attached to an Akta Prime chromatography system (GE Healthcare). The sample was run at 0.4 mL/min in SEC buffer B with 150 mM NaCl, and 300-µL fractions were collected and concentrated using a 100 kDa MWCO Vivaspin2 filter unit (Sartorius, Goettingen Germany) to ~1 mg/mL, and stored at -80°C.

2.2.8 Analysis of the secondary structure of the purified OR subunits by Circular Dichroism (CD) spectroscopy

CD spectroscopic data were collected using a Jasco J-815 circular dichroism spectrophotometer. Samples were provided at a concentration of 0.3 mg/mL and 0.1 mg/mL for DmOrco and DmOr22a respectively in buffer containing 50 mM NaH$_2$PO$_4$ pH 7.5, 150 mM NaCl, and 0.2 mM Zwittergent 3-16. Samples were placed in 0.2 mm cuvettes at 25 °C. The CD spectra obtained were analysed using the SOMCD algorithm (Unneberg et al. 2001) to predict the secondary structure content for each protein.

2.2.9 Preparation of liposome associated OR subunits

Liposomes were prepared using a phospholipid solution produced by evaporating solutions containing: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and cholesterol (CH) at a molar ratio of 5:3:3:1 in a small glass tube under a stream of N$_2$ gas, then desiccating under vacuum for 1 hour. These lipids were resuspended in 1 mL of rehydration buffer (10 mM HEPES pH 7.5, 300 mM NaCl) by vortexing for 5 min followed by sonicating on a Microson ultrasonic cell disrupter (Medisonic, USA) five times at 20% power for 10-20 seconds, placing the sample on ice between each sonication step for 1 min. To promote the formation of liposomes, 10 freeze/thaw steps were performed by transferring
the tube from liquid nitrogen to a 40°C water bath. Liposomes were then sized by passing the lipid solution 11 times through a 100-nm polycarbonate membrane using an Avestin LiposoFAST extruder unit (Avestin, Germany). Glycerol was added at 10% of the final volume and aliquots at 10 mg/mL were snap frozen in liquid nitrogen and stored at -80°C.

Purified OR subunits were reconstituted into the synthetic liposomes in a similar manner to the protocol of Geertsma et al. (2008). Prior to their use, liposomes were defrosted on ice and then destabilised by incubating with 0.2% CHAPS for 15 minutes at room temperature. Then 200 µg of DmOrco was added to 1 mg of liposomes and rotated at 10 rpm for 1 hour at room temperature. Excess detergent was removed by four additions of 25 mg of Bio-Beads SM-2 (Bio-Rad, USA) and incubation at 4°C for 30 min, 2 hours, overnight and a further 2 hours respectively. The Bio-Beads were removed after each incubation period. The DmOrco integrated liposomes were pelleted by centrifugation at 100,000g for 1 hour, and were resuspended in 500 µL of rehydration buffer.

Integration of DmOrco into liposomes was assessed by density gradient ultracentrifugation (DGU) using Accudenz (Accurate Chemical & Scientific Corporation, USA) (Nozawa et al. 2011). The integrated liposomes were brought to 40% Accudenz by the addition of an equal volume of 80% Accudenz solution, placed at the bottom of an ultra-centrifugation tube, and overlaid with 30% Accudenz solution, and DGU buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol). The sample was then centrifuged at 100,000g for 4 hours at 4°C. Liposomes will float to the top of the gradient after Accudenz DGU because of their low density.

For cell-free insertion of protein into liposomes, the wheat germ cell-free reaction was supplemented with 2 µL of 10 mg/mL pre-formed liposomes. Integration was again assessed by DGU using Accudenz as described above.
Chapter 2: Recombinant Expression, Detergent Solubilisation and Purification of ORs

2.3 Results and Discussion

2.3.1 Assessment of different expression systems for OR subunit production

Recombinant production of insect OR subunit proteins was initially attempted using bacterial expression approaches (Miroux and Walker 1996; Lundstrom et al. 2006; Junge et al. 2008; Liu et al. 2012). To improve the chances of success, expression trials were performed using *E. coli* codon-optimised genes and in C43 BL21(DE3) cells, an *E. coli* strain that has been previously used for toxic and membrane protein expression (Miroux and Walker 1996). Three translation enhancing N-terminal peptide tags (Esposito and Chatterjee 2006) were also tested: Maltose binding protein (MBP) (Fox and Waugh 2003; Nallamsetty and Waugh 2006), Peptidyl-prolyl isomerase B (PpiB) (Song et al. 2009; Kralicek et al. 2011), and Thioredoxin (LaVallie et al. 1993; Ishihara et al. 2005). Expression trials performed under the control of T7 and heat-inducible $\lambda$ promoters in pETMCSI and pND706, respectively (Love et al. 1996), resulted in the stalling of cell growth and no evidence of protein production (data not shown). Expression of non-tagged versions of the OR proteins was also attempted under the control of the weaker tac promoter (pMAL2-cx), which has been previously used to over-express a number of membrane proteins and target them correctly to the cytoplasmic membrane of *E. coli* (Drew et al. 2003). Using this promoter, induction did not stall cell growth; however, there was no evidence for recombinant expression on SDS-PAGE or by western blot analysis (data not shown).

Some membrane proteins have been successfully expressed using *E. coli* cell-free protein synthesis systems (Katzen et al. 2009). Translation/solubility enhancing tags can also improve cell-free expression yields (Tsitoura et al. 2010). Cell-free expression of the N-terminally peptide-tagged insect ORs was attempted, with no evidence of full length expression by SDS-PAGE analysis, or with an anti-his$_6$-tag western blot (data not shown). Analysis by anti-MBP western blot showed a ladder of truncated expression products for the MBP tagged constructs, suggesting translation was being stalled on the ribosome (data not shown). In light of these results, it was concluded that neither recombinant *E. coli* cell-based nor cell-free expression was a viable option for recombinant insect OR production.
Two eukaryotic expression systems were then assessed for their ability to produce recombinant insect OR subunits. Both baculovirus-mediated Sf9 cell and wheat germ cell-free expression systems were capable of producing recombinant protein of the predicted molecular weight (MW) for all five proteins examined (Figure 0-1). Membrane proteins expressed in cell-free systems are often produced as precipitants (Goren and Fox 2008); however, the insect ORs expressed in the wheat germ system remained in solution until pelleted under high speed centrifugation (see Chapter 4 Figure 4-6). This is probably because of the presence of plant lipids in the reaction mix (Goren and Fox 2008). Genji et al. (2010) showed that bacteriorhodopsin can be produced in the wheat germ system in a similar fashion; however, without the presence of detergents it was not functional. Kaiser et al. (2008) also showed that the addition of detergents to the wheat germ cell-free reaction increased the solubility of mammalian olfactory receptors. Odorant receptors produced using baculovirus-mediated expression were not produced in a soluble form likely being inserted into the Sf9 cell membrane. It was therefore necessary to solubilise these membrane bound proteins with detergent before purification.

![Western blot analysis of the expression of five odorant receptors from Drosophila melanogaster](image)

**Figure 0-1:** Western blot analysis of the expression of five odorant receptors from *Drosophila melanogaster*. A) wheat germ cell-free expression, and B) baculovirus-mediated expression in insect Sf9 cells. Lanes 1-5 are; DmOr22a, DmOr10a, DmOr35a, DmOr43b and DmOrco, with either an N Terminal Myc (A) or Flag (B) tag.
2.3.2 Effect of different detergents on odorant receptor solubility

Nineteen detergents were chosen for testing for their ability to solubilise insect ORs based on previous studies of membrane proteins expressed in cell-based and cell-free systems (Table 0-2). The properties of these detergents covered a range of critical micelle concentrations (CMC) and ionic properties. These detergents were all investigated for their ability to solubilise the five insect OR subunits after recombinant over-expression in both eukaryotic expression systems. The detergents were tested at 1% w/v, a concentration that is above all the CMC values for the detergents.

Table 0-3 summarises the detergent solubilisation results observed for the five ORs when expressed in both systems. In the baculovirus system, nine of the 19 detergents were capable of solubilising DmOrco, four solubilised DmOr22a and DmOr35a, and only three solubilised DmOr10a and DmOr43b. The wheat germ cell-free expression system produced protein that was much more amenable to solubilisation in a wider range of detergents; fourteen of the detergents were capable of solubilising DmOrco from the wheat germ system to some degree, 13 solubilised DmOr22a and DmOr35a, 11 solubilised DmOr43b, and eight solubilised DmOr10a. An example of the western blot results that were obtained is shown for the DmOr22a subunit in Figure 0-2. None of the detergents was able to solubilise all the protein for any subunit completely in either system. Also, there was no difference in solubility between incubating the protein with detergent for 1 hour compared with 24 hours (data not shown). There was also no effect on protein solubilisation when the detergent concentration was increased to 5% w/v (data not shown).
# Chapter 2: Recombinant Expression, Detergent Solubilisation and Purification of ORs

## Table 0-3: Detergents tested for solubilisation of insect odorant receptor subunits from Sf9 cells and the wheat germ cell-free expression system (WG)

<table>
<thead>
<tr>
<th>Detergent</th>
<th>DmOrco</th>
<th>DmOR10a</th>
<th>DmOR22a</th>
<th>DmOR35a</th>
<th>DmOR43b</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sf9</td>
<td>WG</td>
<td>Sf9</td>
<td>WG</td>
<td>Sf9</td>
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<tr>
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<td>✓</td>
<td>-</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Brij 78</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>Brij 98</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
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<td>✓</td>
<td>-</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
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</tr>
<tr>
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<td>✓</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Zwittergent 3-16</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Lauryl sarcosine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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</tr>
<tr>
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<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
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<td>✓</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Saponin</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triton X-114</td>
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<td>✓</td>
<td>-</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tween 20</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td>Octyl B-D G</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Cholate</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>LDAO</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PEG 8000 (negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>control)</td>
<td></td>
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</tr>
</tbody>
</table>

- ✓ Detergent solubilised the protein to some level
- - Detergent did not solubilise the protein at all
- NT Detergent not tested on this sample
Baculovirus-expressed DmOrco ran as a doublet on western blots (Figure 0-1 B, lane 5). This result could indicate the presence of a glycosylated and a non-glycosylated form. There are two predicted N-linked glycosylation sites on DmOrco, both in extra cellular loop 2 (N169 and N188), one of which (N169) has been shown experimentally to be an acceptor site for N-linked glycosylation (Lundin et al. 2007). DmOrco expressed in the wheat germ cell-free system ran as a single band, presumably because this system does not have the ability to perform post-translational modifications.

The detergents Zwittergent 3-16, sodium dodecyl sulphate (SDS) and lauryl sarcosine were capable of solubilising all OR subunits from either expression system to some extent, whereas the detergents Brij 35, Brij 58, Brij 78 and Brij 98 were capable of solubilising all OR subunits only when expressed in the wheat germ system (Table 0-3). Since SDS and lauryl sarcosine are both strong anionic detergents with the ability to denature proteins at high concentrations, they were not considered suitable for producing soluble material for functional and structural studies.
Chapter 2: Recombinant Expression, Detergent Solubilisation and Purification of ORs

The detergent Zwittergent 3-16 was chosen for subsequent functional and structural studies. Zwittergent 3-16 has previously been utilised for extracting membrane proteins from cells (Mills and Freedman 1983; Squire et al. 1984). It has also been used for separating subunits of lipovittelin (Groche et al. 2000) – a major protein component of egg yolks, and for separating fimbriae and adhesin from E. coli (Hoschutzky et al. 1989). Surprisingly, some of the most commonly used detergents for extracting membrane proteins were not capable of solubilising insect ORs from insect cells, including DDM (Tsitoura et al. 2010; Galian et al. 2011; Mulligan et al. 2012), sodium deoxycholate (Yumen et al. 2012; Lin et al. 2013) and β-octyl-glucopyranoside (Shevchenko et al. 2012).

2.3.3 Large-scale purification of the Drosophila Orco and Or22a subunits

DmOrco and DmOr22a were purified from both expression systems in two steps, first by histag affinity chromatography (Figure 0-3), then by size exclusion chromatography (SEC) (Figure 0-4 and Figure 0-5). During histag affinity purification, the concentration of detergent was reduced from 1% to 0.008%, which is >3x the CMC for Zwittergent 3-16. The first two elution fractions from the Ni-NTA column were pooled and 250 µL was loaded onto the SEC column.
DmOrco was purified from both expression systems ran as a single peak corresponding to a size greater than 200 kDa, with very little presumed to be aggregated protein due to the size of the peak corresponding to the void volume of approximately 7-8 mL for this column (Figure 0-4 A and B). In comparison DmOr22a purified from both expression systems (Figure 0-4 C and 4D) ran as two peaks on the SEC profile, one of which corresponded to very high apparent MW aggregated protein, with the other peak running at an apparent MW of less than 200 kDa. It is not possible to determine the exact size of membrane proteins in detergent micelles from the SEC profile, as the apparent mass of the protein peak includes the associated detergent molecules. However, the fact that DmOrco has a larger MW on
western blots than DmOr22a and runs at a slightly higher apparent MW under SEC does provide some confidence that the proteins are folding into their native quaternary structure. The predicted micelle size of Zwittergent 3-16 is 60 kDa, which would leave ~ 140 kDa of mass allocated to the protein. This again suggests that the proteins are forming quaternary structures in this detergent. Previous studies have shown both homomeric and heteromeric interactions for insect ORs (Neuhaus et al. 2005; Benton et al. 2006; Tsitoura et al. 2010; German et al. 2013), although there are, as yet, no data relating to the stoichiometry of these interactions.

Figure 0-4: Size exclusion chromatography profiles of purified insect odorant receptor subunits. A) DmOrco expressed in Sf9 cells, B) DmOrco expressed in the wheat germ cell-free system, and C) DmOr22a expressed in Sf9 cells, D) DmOr22a expressed in the wheat germ cell-free system. Dashed profiles are standard proteins with MW of 200 kDa, 66 kDa, 29 kDa and 13.7 kDa respectively from left to right. The void volume calculated from blue dextran (2 MDa) is ~7.5 mL.
2.3.4 Secondary structure analysis of the purified DmOrco and DmOr22 subunits

Circular dichroism spectroscopy was performed on the detergent-solubilised DmOrco and DmOr22a subunits to determine if they were correctly folded. The CD spectra were analysed using the SOMCD programme (Unneberg et al. 2001) to calculate the secondary structure of each protein. DmOrco was predicted to contain 75% α-helix, 5% β-sheets, 6.4% turns and 13.6% random coil. DmOr22a was predicted to contain 79% α-helix, 3.2% β-sheets, 6% turns and the rest is predicted to be random coil (Figure 0-6). The CD measurements show a high degree of secondary structure suggesting both the DmOrco and DmOr22a subunits are folded correctly.
Chapter 2: Recombinant Expression, Detergent Solubilisation and Purification of ORs

Figure 0-6: Circular Dichroism spectra of detergent solubilised OR subunits. CD spectroscopic data were collected on 0.3 mg/mL DmOrco (red line) and 0.1 mg/mL DmOr22a (blue line) in 50 mM NaH$_2$PO$_4$ pH 7.5, 150 mM NaCl, and 0.2 mM Zwittergent 3-16. Buffer scans were subtracted from protein scans and data are presented as Mean residue ellipticity.

2.3.5 Insertion of DmOrco and DmOr22a into liposomes

Peak fractions collected from the SEC for DmOrco expressed in Sf9 cells could be concentrated to ~2 mg/mL with no change in the resulting SEC profile, indicating that they are capable of being concentrated without aggregation (data not shown). Yields of DmOrco were estimated to be ~5-6 mg per litre of Sf9 cells, and ~0.3 mg per mL of WG reaction.

Purified DmOrco from baculovirus-mediated expression was inserted into reconstituted liposomes following a modified protocol of Geertsma et al. (2008), with an Accudenz density gradient used to assess the level of integration of recombinant protein into liposomes (Figure 0-7). Western blots showed that when DmOrco was subjected to density gradient ultracentrifugation without the presence of liposomes, the protein was concentrated at the bottom of the gradient, with some protein distributed throughout (Figure 0-7 A). When DmOrco was integrated into liposomes, they float to the top three fractions of the gradient (Figure 0-7 B). Wheat germ expressed material was also successfully inserted into liposomes.
using a protocol from Nozawa et al. (Nozawa et al. 2007), whereby the pre-formed liposomes were added directly to the wheat germ reaction (data not shown). The successful reconstitution of protein into artificial membranes is a critical step towards future structural and functional studies on this important group of insect odorant receptor proteins.

Figure 0-7: Accudenz density gradient centrifugation of DmOrco and DmOrco inserted into liposomes. Western blots of DmOrco in the absence (A) and presence (B) of liposomes. Lanes 1-8 are fractions taken from the bottom to the top of the gradient. Note the evidence of potential dimer formation in the presence of liposomes.

The ability to recombinantly express, detergent-solubilise and purify integral membrane protein subunits is a necessary prerequisite for studying the structure and function of receptor complexes in vitro. In turn, the development of these procedures allows downstream studies of receptor complex composition, subunit stoichiometry, quaternary structure and activation mechanism using in vitro approaches. I have developed expression, detergent solubilisation and purification protocols for subunit members of the insect odorant receptor complex, a large family of ligand-gated cation channels for which such procedures are currently not available. My efforts have focused on the obligate receptor subunit, DmOrco, and four other ligand-binding receptor subunits, DmOr10a, DmOr22a, DmOr35a and DmOr43b, from D. melanogaster. Two eukaryotic expression systems were successfully employed to produce recombinant insect OR subunits, namely the baculovirus expression system and a wheat germ cell-free expression system. The baculovirus expression system was amenable to scale up and is capable of producing milligram
quantities of protein from a litre of cells. On the other hand, the wheat germ expression system is limited to smaller volumes of production using the analytical scale methods we employed, although the recombinant material was more easily solubilised by a number of detergents relative to the material produced in the baculovirus expression system. The Zwittergent 3-16 was capable of solubilising all subunits from both expression systems and maintaining the subunits in a similar form through two purification procedures. The successful purification of insect OR subunits and their integration into artificial membranes paves the way for further functional and structural analysis of this important and novel family of receptors, which I pursue in subsequent chapters of this thesis.
3

Investigation of the Oligomeric Structure and Interactions between Insect OR Subunits
3.1 Introduction

3.1.1 Insect Odorant Receptor complex

In odorant sensory neurons, insect odorant receptor subunits have been proposed to form an odorant receptor complex that is believed to act as a non-selective ligand-gated cation channel. Virtually all known ion channels form oligomeric structures of either homomeric or heteromeric complexes. Therefore, an initial hypothesis to start from is that insect odorant receptors also form similar oligomeric complexes. Whilst there is evidence demonstrating that Orco acts as an ion channel when expressed alone (Jones et al. 2011), the ligand-binding OrX subunits also affect the structure of the ion channel, or at least modify the ability of reagents such as Ruthenium red to inhibit the channel (Pask et al. 2011). These data indicate that the channel might be either homomeric or heteromeric depending on the concentration of each subunit. Insect odorant receptor subunits are also known to interact with Orco in vivo to enable each OrX subunit to localize to the dendritic membrane (Benton et al. 2006).

Resonance energy transfer (RET) experiments have demonstrated that insect OR subunits interact in heterologous cells systems to form both homomeric and heteromeric complexes (Neuhaus et al. 2005; German et al. 2013). Pull down assays using protein expressed in insect cells have also demonstrated an interaction between mosquito Orco and OrX subunits (Tsitoura et al. 2010). The site of this interaction is likely to be in the third intracellular loop, as shown by yeast-2-hybrid experiments with DmOrco, DmOr43b and DmOr22a (Benton et al. 2006). However, there is no literature defining the stoichiometry of subunits within the complex.

The ligand binding ORs are also capable of inducing a calcium response to ligands when expressed without Orco in heterologous cells (Wetzel et al. 2001; Sakurai et al. 2004; Grosse-Wilde et al. 2006). These experiments indicate that the OrX subunit is likely to be interacting with a co-expressed Gα protein, which is in turn interacting with endogenous secondary messenger systems to open calcium channels. The fact that the OrX subunit is capable of interacting with second messenger systems in heterologous cells begs the
question of whether they are interacting directly with Orco in vivo. Perhaps they are interacting with Gα proteins, which are then activating the Orco ion channel (Nolte et al. 2013). Perfusion of pheromone sensitive sensilla in the Hawkmoth (Manduca sexta) with VUAA1 did not increase pheromone responses, but did increase spontaneous activity in tip-recordings, indicating that at least in this insect, Orco appears to be a second-messenger dependent channel (Nolte et al. 2013).

Understanding the possible oligomeric structure of both the homomeric subunits and the heteromeric complex will address questions concerning the structure of the proposed ion channel and the roles that each of the subunits has to play in insect olfaction. As Orco must interact with each of the ligand binding OrXs to traffic these subunits to the dendritic membrane (Benton et al. 2006), it is highly likely that the stoichiometry of the complex is stable across the OR family, and potentially Class Insecta.

3.1.2 Insect ORs are likely to be related to Potassium channels

The insect OR complex has been proposed to be a non-selective ligand-gated cation channel, likely related to potassium channels (Benton et al. 2006). The ability of insect ORs to form both homomeric and heteromeric interactions is a characteristic that is shared with many families of potassium channels. Another shared characteristic is the ability of subunits from different species to interact and form functional complexes (Li et al. 1992). However, the stoichiometry of potassium channels is well understood with every known example forming a functional tetramer, and a well described tetramerisation domain evident in many families (Choe 2002). There is no evidence for such a domain in the insect ORs and as yet no evidence supporting a tetrameric structure. However, there is evidence that the functional OR complex is an ion channel made up of at least two subunits Orco and OrX (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008; Jones et al. 2011; Pask et al. 2011; Chen and Luetje 2012; Jones et al. 2012).

The majority of potassium channels are made up of four monomers each with two to six transmembrane (TM) domains and a pore loop that forms the selectivity filter. However, the BK channels have 7 TMs similar to the insect Ors, but with the opposite orientation in the membrane like that of GPCRs with an extracellular N terminus. The selectivity filter domain
of potassium channels is conserved across nearly every family, a domain very similar to this is found in TM6 of Orco and has been postulated to perform a similar function in the OR complex (Wicher et al. 2008). Mutagenesis of this domain in D. melanogaster Orco resulted in a reduction in the K⁺ permeability in Xenopus laevis oocytes (Wicher et al. 2008).

In the Shaker-type potassium channels there is evidence that the stoichiometry of the subunits that make up the heteromeric channel can directly influence the channel properties (Xu et al. 1998). The four pore-forming α subunits provide four interaction sites for the Kvβ subunits of which there are two types Kvβ1 and Kvβ2. The Kvβ1 subunit can interact in an α₄βₙ stoichiometry where n = 0, 1, 2, 3 or 4 depending on the concentration of subunits, however the Kvβ2 subunit forms homomers and only interact with the α subunits in an α₄β₄ stoichiometry. This variation in stoichiometry provides a molecular mechanism to alter the function of the ion channel (Xu et al. 1998).

If the insect ORs do resemble potassium channels, the insect OR ion channel should also be made up of four subunits. This channel could be a homomeric Orco channel that interacts with OrX in some way, or it could have a range of stoichiometries from 3 Orco subunits and 1 OrX, to 3 OrX subunits and 1 Orco (Figure 0-1 B-D). The α₄βₙ stoichiometry would provide a useful mechanism to alter the function of the insect OR ion channel. Taking the Orco subunit to be the homologue of the α subunit and forming the pore of the channel (Figure 0-1 A), there would potentially be four separate interaction sites for the OrX subunit to bind to which could result in four different odorant binding patterns depending on the number of OrX subunits in the OR complex (Figure 0-1 E-H).
Chapter 3: Investigation of Oligomeric Structure and Interactions between OR Subunits

Figure 0-1: Potential ion channel stoichiometry of the insect Ors. If the insect OR channel is assumed to form a tetramer like the K⁺ channels, they should be formed of either homomeric Orco (A) or a combination of Orco and OrX subunits (B-D). If the ORs follow the αβn stoichiometry of shaker potassium channels they could potentially form any of the stoichiometries shown in E-F. Red circles indicate Orco subunits, and blue circles indicate OrX subunits.

However, if the insect OR complex does not form a structure similar to that of K⁺ channels there are an almost unlimited number of multimeric and stoichiometric variants that could be formed. Thus, understanding the oligomeric structure of the subunits in the complex is an important step in understanding the structure and function of the insect OR complex.

If the insect ORs are forming a stable complex in vivo then it should be possible to reconstitute this complex from purified subunits (Berrier et al. 2004; Asmar-Rovira et al. 2008; Genji et al. 2010; Giudici et al. 2013).

3.1.3 Techniques for the Analysis of Oligomeric Structure in Membrane Proteins

3.1.3.1 Native Page Gels

Native PAGE analysis is a common technique used to investigate the oligomeric state of membrane proteins. It allows the separation of potential higher order structures based on their molecular weights without the need to denature them first, allowing analysis of their
native states. The motility of the protein complexes is dependent on their size and charge allowing smaller molecular weight protein to move faster and thus further through the gel. Native PAGE gels have been used to demonstrate higher order structures in KcsA (Giudici et al. 2013), Porins (Freixeiro et al. 2013) and potassium channels (Ramjeesingh et al. 1999), and have also been used to assess aggregation states of membrane proteins prior to crystal trials (Ma and Xia 2008). The choice of detergent will again need to be investigated as it may have an effect on the migration pattern of proteins in the gel matrix.

However, there are some problems associated with the use of detergent-based buffers in Native PAGE. The mobility of the protein through the gel matrix is dependent on the addition of a negative charge to the protein commonly through the use of Coomassie blue dye. The size of the detergent micelle can affect the amount of protein available for the Coomassie dye to bind to, and some detergents can impart a charge on the protein themselves. Therefore the choice of solubilising detergent can again be a critical factor in the analysis of the oligomeric states of membrane protein with Native PAGE (Reisinger and Eichacker 2008).

3.1.3.2 Cross-linking

Chemical cross-linking stabilizes interactions between proteins through covalent bonds. This allows the detection of protein-protein interactions that may be transient or weak in nature. Disuccinimidyl glutarate (DSG) and disuccinimidyl suberate (DSS) are homobifunctional cross-linkers that possess identical reactive groups at the end of a spacer arm; however DSG has a 7.7 Å linker, while DSS has an 11.4 Å linker separating amine-reactive N-Hydroxysuccinimide (NHS) esters that can crosslink amine groups at the N-terminus of peptide chains and on the side chain of lysine residues (Hari et al. 1987; Pimenova et al. 2008; Madler et al. 2010; Chavez et al. 2011). They are both cell membrane permeable and can be used to crosslink proteins in vivo. Bis[sulfosuccinimidyl] suberate (BS3) is a water soluble homobifunctional cross-linker that is not cell membrane permeable. It has an 11.4 Å linker separating amine reactive Sulfo-NHS esters capable of cross-linking the same residues as DSS and DSG (Dihazi and Sinz 2003; Fritzsche et al. 2012). This compound is particularly useful for cross-linking cell surface proteins. BMPA (N-ß-Maleimidopropionic acid) contains
both maleimide and carboxyl reactive groups, which are reactive toward sulfhydryls and hydrazides/amines, respectively, conferring the ability to crosslink cysteine residues as well as carboxyl residues at the C-terminus of the peptide chain and the side chains of aspartic acid and glutamic acid. The potential complexes can be inferred by comparing untreated protein with the cross-linked protein on SDS-PAGE.

3.1.3.3 Size Exclusion Chromatography – Multi Angle Light Scattering (SEC-MALS)

One method to examine the stoichiometry of protein complexes is to measure the molecular weight (MW) of the complex and then use the known MW of the monomers to deconvolute the ratio of the subunits. Estimating the MW of a membrane protein can be difficult with standard techniques designed for soluble proteins. Membrane proteins need to be maintained in a detergent solubilised form and so the mass of the detergent bound to the protein needs to be taken into account. Standard gel filtration columns that are used in size exclusion chromatography (SEC) cannot accurately estimate the mass of the protein detergent complex as the movement of the complex through the column resin is not dependent on the size of the protein. Standard HPLC setups are generally equipped with a single UV detector that is used to measure the amount of protein coming off the end of the column; however when other detectors are added in series with the UV it is possible to more accurately measure the mass of the eluted protein.

There are only two classical techniques that allow the direct measurement of the absolute molecular mass of a protein that is in a detergent solubilised form. These are sedimentation equilibrium centrifugation and static light scattering (Slotboom et al. 2008). Sedimentation equilibrium centrifugation requires the use of high-end centrifuges that are not available in New Zealand. The use of multi-angle light scattering (MALS) and refractive index (RI) sensors provides the capability of determining the absolute molecular weights of proteins and complexes in detergent micelles. Molecular weights determined by SEC-MALS are independent of the SEC elution position, and problems such as non-globular shape and interaction with the SEC support, which can pose limitations on the use of SEC for estimating MW, have no impact on MWs determined by SEC-MALS (Folta-Stogniew and
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Williams 1999; van Dijk and Smit 2000). SEC-MALS is also a non-invasive technique as it does not require the incorporation of a radioactive or fluorescent tag, it is also non-destructive, and the samples may be recovered for use in subsequent studies. Compared with techniques such as analytical centrifugation, SEC-MALS is rapid, and allows for the analysis of samples in buffers of various pH values, ionic strengths, and temperatures and in the presence or absence of ligands (Folta-Stogniew and Williams 1999). This approach has been used to determine the oligomeric state of an ATP-independent periplasmic transporter (Mulligan et al. 2012), and the hexameric state of a UREA channel (Huysmans et al. 2012).

3.1.3.4 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical technique that allows real-time monitoring of biomolecular interactions (Cooper 2002). This method utilises the movement of surface plasmons resonating across a metallic surface (usually gold) (Figure 0-2). Surface plasmons are electromagnetic waves that move across the surface of a chip in a pathway that is parallel to the chip and the environment that it is interacting with (e.g. liquid, gas, lipids). These waves are extremely sensitive to mass changes on the surface of the chip, such as the binding of other proteins and ligands to proteins that have been immobilised on the chip surface.
Figure 0-2: Typical set-up for an SPR biosensor. Surface plasmon resonance (SPR) detects changes in the refractive index close to the surface layer of a sensor chip. SPR is observed as a sharp shadow in the reflected light from the surface at an angle that is dependent on the mass of material at the surface. The SPR angle shifts (from I to II in the lower left-hand diagram) when biomolecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored in real time as a plot of resonance signal (proportional to mass change) versus time (lower right hand diagram). Modified from Cooper (2002).

SPR allows for the measurement of direct interactions between protein subunits, and also potentially between receptors and their ligands. SPR is a powerful tool for determining the binding kinetics of proteins in a complex and as such allow rapid analysis of differences between each Orco-OrX complex, as well as identifying the potential interaction and ligand-binding sites through mutagenesis of the receptors. The inherent difficulties involved with purifying and maintaining membrane proteins in solution have meant that the analysis of membrane proteins with SPR has lagged behind that of soluble proteins and antibodies (Rucker et al. 2010; Rich et al. 2011). However, in recent years there have been advances in both the composition of the chips and immobilisation techniques that have allowed researchers to successfully bind membrane proteins to the SPR surface and analyze both protein-protein and protein-ligand interactions.
The major hurdle that has to be overcome prior to carrying out SPR experiments appears to be the production of protein in a format that can be immobilised on the chip in a functional state. The most common approach used to immobilise membrane proteins is via antibodies bound to the chip surface, and running buffers containing a detergent to mimic a membrane environment. The choice of antibody is varied with His$_6$ (Borch et al. 2011; Christopeit et al. 2011), 1D4 (Navratilova et al. 2005; Navratilova et al. 2006; Navratilova et al. 2006; Rich et al. 2009; Christopeit et al. 2011; Rich et al. 2011), Myc (Benilova et al. 2008), and Flag (Borch et al. 2011) antibodies all able to be used.

The methods used to produce proteins for SPR also vary, with wheat germ (Kaiser et al. 2008) and E. coli cell-free systems, as well as mammalian cells (Stenlund et al. 2003; Cook et al. 2009; Rich et al. 2009; Rich et al. 2011), insect cells (Sen et al. 2005; de Kloe et al. 2010; Christopeit et al. 2011; Congreve et al. 2011; Rich et al. 2011), E. coli (Harding et al. 2006; Harding et al. 2007; Ohvo-Rekila and Mattjus 2011; Tikhonova et al. 2011), and yeast (Vidic et al. 2006; Benilova et al. 2008) all being employed. Although it appears to be beneficial to use pure protein where possible, some examples show that crude cell lysates can be flowed across the SPR chip to achieve sufficient immobilisation for both protein-protein and protein-ligand binding studies (Rich et al. 2009; Congreve et al. 2011). SPR has also been used to screen solubilisation, purification, and crystallisation conditions for membrane proteins (Navratilova et al. 2005; Navratilova et al. 2006).

### 3.1.4 Aims

In Chapter 2 I demonstrated that the insect OR subunits can be over-expressed from different expression systems, solubilised and purified. The resulting purified receptors have a predominantly α-helical secondary structure similar to many membrane proteins. Within this chapter I aim to use the purified OR subunits to investigate the hypothesis; that the DmOrco and DmOrX subunits are capable of forming homomeric and heteromeric oligomers.

To test this hypothesis I will use three methods that have proven successful in determining the oligomeric nature of membrane proteins previously.
1) I will examine the banding patterns of the subunits on Native PAGE gels looking for evidence that would suggest oligomers are forming. Although these subunits appear to be forming mostly homogenous peaks on SEC, the profiles can be misleading due to the presence of detergents in the samples and buffers.

2) I will determine the absolute molecular weight of the subunits post SEC purification using SEC-MALS. This will provide insights into the exact size of the oligomeric structures and should allow the calculation of the number of monomers in the complex.

3) I will investigate the utility of SPR to determine the binding kinetics of insect OR subunits. The BIAcore SPR system allows the calculation of the on and off rates of any subunit interactions, and the stability of the complexes formed under various conditions. These experiments will be carried out in a similar fashion as the pull-down experiments, whereby one subunit will be immobilised on the chip surface and the other subunits will be passed over it. An interaction will be indicated by a change of mass on the chip surface indicated by an increase in RU (arbitrary reference units) that does not return to baseline.
3.2 Materials and Methods

3.2.1 Expression and purification of insect OR subunits

Five insect odorant receptor subunits (DmOr10a, DmOr22a, DmOr43b, DmOr67d and DmOrco) were expressed and purified as described in section 2.2.7 using baculovirus-mediated expression in Sf9 cells with slight modifications. The culture volumes were increased to 500 mL and the purification was carried out on a 5 mL Ni-NTA column and a HiLoad 16/60 Superdex 200 SEC column. The subunits were concentrated to 1.5-2.8 mg/mL with a 100,000 molecular weight cutoff (MWCO) centrifugal concentrator (Vivaspin) and stored at -80°C in buffer containing 50 mM NaH₂PO₄, 150 mM NaCl, 0.2 mM Zwittergent 3-16.

Wheat germ cell-free reactions were carried out as described in section 2.2.4.2 and used fresh.

3.2.2 Native PAGE gels

Native PAGE experiments were carried out as per Life Technologies recommendation with modifications according to Ramjeesingh (1999). The purified receptors were mixed with an equal volume of 2 x loading buffer (100 mM Tris pH 8.0, 20% glycerol and 0.02% bromophenol blue), detergent was added to a final concentration of 4% (Ramjeesingh et al. 1999), and 6 µg of each subunit was loaded on a Native PAGE Novex 4-16% Bis-Tris gel (Life Technologies). The samples were loaded as soon after thawing as possible and were not vortexed or heated. Electrophoresis was carried out at 150 V for 1 hour at 4°C, then 250 V for a further hour at 4°C. The gels were Coomassie stained according to the manufacturers’ protocol (Life Technologies).

3.2.3 Cross-linking experiments

The four cross-linking reagents (Disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), Bis[sulfo]succinimidyl] suberate (BS3) and N-ß-Maleimidopropionic acid (BMPA)) were purchased from Proteochem (WY, USA) and made up to 50 mM in DMSO as per the manufacturer’s instructions.
3.2.3.1 Cross-linking experiments with purified DmOrco subunit

Purified His\textsubscript{10}-Flag-DmOrco was defrosted from the -80°C stock and brought to room temperature rapidly. The recommended ratio of 20:1 cross-linker to protein was used for initial experiments. Reactions were set up with 35 µM protein and 0.77 mM of each cross-linker. The samples were incubated at room temperature for 1 hour then the reaction was quenched with 50 mM Tris pH 7.5 for 15 minutes at room temperature. Evidence of the formation of higher order structures was assessed by Coomassie stained SDS-PAGE as described in section 2.2.5.

3.2.3.2 Cross-linking experiments with purified OR subunits reconstituted into liposomes

To examine the possibility that the OR subunits will form either heteromeric or homomeric complexes when reconstituted into an artificial membrane, two subunits (His\textsubscript{10}-Flag-DmOrco and His\textsubscript{10}-Myc-DmOr43b) were inserted into preformed liposomes either separately or together as described in section 2.2.9. For each cross-linking reaction, 1 µL of each reagent was added to 9 µL of proteoliposomes to give a final concentration of 5 mM for each reagent. The samples were incubated at room temperature for 1 hour and the reaction was quenched with 50 mM Tris pH 7.5 for 15 minutes at room temperature. Evidence of the formation of oligomeric structures was assessed by SDS-PAGE as described in section 2.2.5.

3.2.4 Size Exclusion Chromatography – Multi Angle Light Scattering (SEC-MALS)

3.2.4.1 SEC-MALS analysis of insect Ors

The SEC-MALS system consisted of a Superdex 200 10/300 GL column (GE healthcare), attached to a Dionex HPLC with DAD-3000 diode array UV/VIS. The MALS detector was a SLD-7000 with a thermostated sample cell attached to a Shodex RI-101 Refractive Index (RI) detector.

The SEC column was calibrated in buffer containing 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, with the respective detergent at 3 x CMC. Bovine serum albumin (BSA) was used as a standard to
avoid the need to determine the absolute refractive index of each buffer and protein complex. This also prevents small errors in the calibration constants of the instruments from being propagated through the molecular mass calculations (Folta-Stogniew 2006).

Purified insect ORs were injected in 100 µL aliquots at 0.5 mL/min at room temperature. For each protein either two or three different concentrations were used starting with the highest concentration available, followed by a 2-3 fold dilution (the concentration varied for each subunit). Analysis was carried out using the WinGPC unichrom software (Polymer Standards Service) and Microsoft Excel, using the three detector method described in Slotboom et al. (2008).

3.2.5 Investigation of subunit interactions using Surface Plasmon Resonance (SPR)

Insect odorant receptors were thawed rapidly from -80°C immediately prior to the experiments. The wheat germ cell-free reactions were prepared fresh and used immediately. Experiments were carried out on a BIAcore T200 or a BIAcore Q machine, and analysed with the BIAcore Q evaluation software.

3.2.5.1 Immobilisation of wheat germ cell-free expressed OR subunits on a Ni-NTA Chip

The series S Sensor Chip NTA (BIAcore) was activated with 50 mM NiCl in a running buffer (50 mM HEPES, 150 mM NaCl, 50 µM EDTA pH7.5) supplemented with 0.1% DDM. The cell-free reactions were incubated with 2% DDM for 30 minutes and diluted 1:5 with running buffer prior to injection at 5 µL/min for 4 minutes. The surface was regenerated with 0.35 M EDTA and 50 mM NAOH.

3.2.5.2 Immobilisation of cell-free expressed subunits using Flag and Myc antibodies

The CMS chip (BIAcore) surface was activated with N-Hydroxysuccinimide (NHS) and ethyl (dimethylaminopropyl) carbodiimide (EDC) at 5 µL/min for 20 minutes. Primary antibody (anti-myc (Santa-Cruz Biotechnology) or anti-flag (SigmaAldrich)) was diluted to 1 mg/mL in
running buffer and injected at 5 µL/min for seven minutes. The cell-free reactions were incubated with 2% DDM for 30 minutes and diluted 1:5 with running buffer prior to injection of 5 µL/min for 4 minutes. Regeneration conditions that were trialed included; 0.1 M glycine at pH 2-5; 0.1 M triethanolamine pH 11.5; Formic acid pH 2; 50 mM NaOH; 3 M MgCl₂; and 25-50% ethylene glycol.

3.2.5.3 Immobilisation of purified subunits using secondary antibody and primary antibodies

Due to the difficulties experienced regenerating the protein bound to primary antibodies a two antibody approach was tested. This involved covalently binding a secondary antibody to the chip surface and immobilising a primary antibody on this, then capturing the protein with an epitope tag recognised by the primary antibody. Regeneration conditions to remove the primary antibody were then trialled.

The CM5 chip surface was activated by flowing N-Hydroxysuccinimide (NHS) and ethyl (dimethylaminopropyl) carbodiimide (EDC) at 5 µL/min for 20 minutes. Secondary antibody was then bound to the activated surface at 100 µg/mL for 24 minutes at 5 µL/min. The surface was blocked with ethanolamine for 10 minutes. Primary antibody (anti-Myc or anti-Flag) was injected at 1 mg/mL for three minutes at 20 µL/min. The receptor subunits were diluted to 100 µg/mL in running buffer supplemented with 0.2 mM Zwittergent 3-16, and injected at 20 µL/min for three minutes. The chip surface was regenerated with one 20 µL injection of 10 mM glycine pH 2, followed by one 10 µL injection of 50 mM NaOH.
3.3 Results

3.3.1 Purification of the Insect OR subunits

Although each of the subunits could be purified almost to homogeneity as determined by SEC and Coomassie stained gels (Figure 0-5 and Figure 0-3), not all formed a single homogenous peak when analysed by SEC (Figure 0-3). Where the subunits produced more than one peak, the fractions from the centre of each peak were concentrated and analysed using Native Page and SEC-MALS (Figure 0-3 C).

The OrX subunits had a larger proportion of protein eluting at the void volume for the SEC column (45 mL) than DmOrco (Figure 0-3). This indicates that there is a much larger proportion of the protein forming aggregates for the OrX subunits compared to DmOrco (Figure 0-3).
Figure 0-3: SEC profiles for each of the DmOR subunits. A) DmOR10a, B) DmOr22a, C) DmOr43b, D) DmOr67d, E) DmOrco. The blue line is the profile for the OR subunit, the dotted line indicates MW standards with peaks from left to right corresponding to; 444 kDa, 200 kDa, 66 kDa, 29 kDa and 13.7 kDa. The left Y axis is the UV A280 for the OR subunit, the right Y axis is the UV A280 value for the standards. The void volume for this column is calculated to be 45 mL using a 2000 kDa blue dextran polymer. Note that the scale of the left hand Y axes vary between subunits. Horizontal lines above each peak indicate fractions pooled and concentrated for further analysis.

3.3.2 Native PAGE analysis

Standard proteins of known molecular weight (NativeMark Protein Standards (Life technologies)) were first run on the NativePage Novex 4-16% Bis-Tris gels and a molecular weight calibration curve was then calculated by plotting the log(MW) of these known standards against the distance of their respective band from the running front of the gel (Rf) (Figure 0-4). A linear regression calculated from this plot allows the calculation of the MW of
the receptor subunits based on their mobility distance from the Rf (Figure 0-4) (Ramjeesingh et al. 1999).

![Graph](image-url)

Figure 0-4: The mobilities of the standard molecular weight proteins relative to the front (Rf) were plotted against the logarithm of their molecular masses (MW), indicated above each data point.

Insect ORs were prepared with six different detergent conditions prior to electrophoresis on 4-16% Native PAGE Bis-Tris gels (Life Technologies) (Figure 0-5). The first condition was the 0.2 mM (0.008%) Zwittergent 3-16, which is the condition that the subunits had been purified in (Section 2.2.7). In this condition DmOrco migrated through the gel in a manner that allowed analysis of the banding pattern (Figure 0-5 A lane I). Multiple bands were observed for DmOrco under this condition. When five different detergents were added to a final concentration of 4% w/v, the banding pattern changed. The addition of Zwittergent 3-16, DDM, or Brij 35 resulted in smearing on the gel and no obvious bands. However, when perfluoro-octanoate acid (PFO) or SDS are added there are obvious bands that are similar to that observed with 0.2 mM Zwittergent 3-16. The major difference between these two detergents appears to be the lack of a 16-mer band in the PFO sample, and the different migration patterns of bands three and four (Figure 0-5 A lanes I, IV and VI).

The ligand-binding subunits only showed discrete bands on the gel when either PFO or SDS detergents were added (Figure 0-5 B-F), although the 0.2 mM Zwittergent 3-16 treatments did show some banding for DmOR43b P1 there was a large amount of background making the bands difficult to define (Figure 0-5 D lane I). The other detergent treatments again
resulted in a smear on the gels. The DmOr10a subunit had four obvious bands in PFO but only two in SDS. The DmOr22a subunit had six bands in both PFO and SDS (Figure 0-5 C lanes IV and VI); however, the bands had different migration patterns in the two conditions. Two different DmOr43b samples were analysed, corresponding to the first (P1) and second (P2) peak on the SEC profile (Figure 0-3 C). The two samples generated a very similar banding pattern of three bands in PFO and two or three in SDS respectively. The P1 sample had four observable bands in 0.2 mM Zwittergent 3-16, but the P2 sample was not able to be analysed in this condition due to the level of smearing observed. The DmOr67d subunit appeared to have a large amount of aggregated protein on the SEC profile (Figure 0-3 D), as the peak has a shoulder that is sloping to the void volume with no clearly defined peak. However, fractions under the highest point of the profile at 70 to 75 mL were collected and concentrated. When this sample was run on the Native Page gel there was only one obvious band in the SDS sample, and no bands in the other conditions.

The molecular weight of each of the observable bands (indicated by a number in Figure 0-5) was analysed by comparing with the migration pattern of the standard proteins (Figure 0-4), and are described in Table 0-1.
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Figure 0-5: Native PAGE analysis of five insect OR subunits. A) DmOrco, B) DmOr10a, C) DmOr22a, D) DmOr43b P1, E) DmOr43b P2, F) DmOr67d. Each receptor was investigated in six different conditions indicated by the lane numbers; I) 0.2 mM Zwittergent 3-16, II) 4% Zwittergent 3-16, III) 4% DDM, IV) 4% PFO, V) 4% Brij 35, VI) 4% SDS. Discrete bands for each sample are numbered and the respective MWs are described in Table 3-1.
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Table 0-1: Estimated molecular weights of the numbered bands from the Native Page gels in Figure 0-5. Theoretical MWs are in brackets for each predicted oligomer. (--) indicates no band was observed on the Native PAGE gel for this predicted MW for this OR subunit.

<table>
<thead>
<tr>
<th>OR</th>
<th>detergent</th>
<th>1 (monomer)</th>
<th>2 (dimer)</th>
<th>3 (trimer)</th>
<th>4 (tetramer)</th>
<th>5 (hexamer)</th>
<th>6 (hexadecamer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmOr10a</td>
<td>PFO (4%)</td>
<td>50.5</td>
<td>101</td>
<td>151.5</td>
<td>202</td>
<td>303</td>
<td>808</td>
</tr>
<tr>
<td></td>
<td>SDS (4%)</td>
<td>-</td>
<td>80.3</td>
<td>180.7</td>
<td>242.1</td>
<td>-</td>
<td>891.4</td>
</tr>
<tr>
<td>DmOr22a</td>
<td>PFO (4%)</td>
<td>(49.9)</td>
<td>(99.8)</td>
<td>(149.7)</td>
<td>(199.6)</td>
<td>(299.4)</td>
<td>(798.4)</td>
</tr>
<tr>
<td></td>
<td>SDS (4%)</td>
<td>-</td>
<td>-</td>
<td>171.4</td>
<td>-</td>
<td>-</td>
<td>804.0</td>
</tr>
<tr>
<td>DmOr43b P1</td>
<td>PFO (4%)</td>
<td>(50)</td>
<td>(100)</td>
<td>(150)</td>
<td>(200)</td>
<td>(300)</td>
<td>(800)</td>
</tr>
<tr>
<td></td>
<td>SDS (4%)</td>
<td>-</td>
<td>118.6</td>
<td>161.2</td>
<td>213.0</td>
<td>312.6</td>
<td>741.6</td>
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<tr>
<td>DmOr43b P2</td>
<td>PFO (4%)</td>
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<td>(100)</td>
<td>(150)</td>
<td>(200)</td>
<td>(300)</td>
<td>(800)</td>
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<td>152.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DmOr67d</td>
<td>PFO (4%)</td>
<td>-</td>
<td>125.0</td>
<td>-</td>
<td>213.2</td>
<td>316.5</td>
<td>905.2</td>
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<td></td>
<td>SDS (4%)</td>
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<td>84.3</td>
<td>166.9</td>
<td>245.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DmOrco</td>
<td>PFO (4%)</td>
<td>(47.7)</td>
<td>(95.4)</td>
<td>(143.1)</td>
<td>(190.8)</td>
<td>(286.2)</td>
<td>(763.2)</td>
</tr>
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<td>SDS (4%)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>822.7</td>
</tr>
<tr>
<td>DmOrco</td>
<td>PFO (4%)</td>
<td>(57.6)</td>
<td>(115.2)</td>
<td>(172.8)</td>
<td>(230.4)</td>
<td>(345.6)</td>
<td>(921.6)</td>
</tr>
<tr>
<td></td>
<td>SDS (4%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>840.9</td>
</tr>
</tbody>
</table>

3.3.2.1 Cross-linking Purified DmOrco

DmOrco that had been purified as described in section 2.2.7 was incubated with each of four cross-linking reagents (DSS, DSG, BS3, and BMPA) at three concentrations; 1 mM, 3 mM and 5 mM. The banding pattern for purified DmOrco by SDS-PAGE indicates one major band at between 40 and 50 kDa, and two bands at ~80 kDa and 110 kDa respectively that are much fainter. This banding pattern did not change when the samples were incubated with cross-linking reagents and visualised by SDS-PAGE (Figure 0-6). However, the samples that had been incubated with BMPA did look different than those incubated with the other cross-linking reagents. The monomeric DmOrco band appeared ‘crisper’ and there was a smear of potentially aggregated protein above this (Figure 0-6, last three lanes), which was
not evident in the other samples. The DmOrX subunits were prone to precipitation when incubated at room temperature and therefore were not able to be cross-linked following the protocol described for the DmOrco subunit supplied by the manufacturer (Proteochem).

![Figure 0-6: 4-12% SDS-PAGE analysis of the cross-linked DmOrco subunit. Purified DmOrco was incubated with 1 mM, 3 mM or 5 mM of each crosslinking reagent (indicated by lane numbers). The first lane is untreated (U).]

### 3.3.2.2 Cross-linking of DmOrco and DmOr43b subunits reconstituted into liposomes

In order to determine if OR subunits were more likely to interact in a membrane-like environment, two subunits (His\textsubscript{10}-Myc-DmOrco and His\textsubscript{10}-Flag-DmOr43b) were reconstituted into preformed liposomes individually, and together, as described in section 2.2.9 (Figure 0-7). Analysis of the fractions from the bottom of the tube after density gradient ultracentrifugation demonstrated that each subunit was present in the predicted liposome containing fraction near the top of the gradient (Figure 0-7 A and B lane 7) indicating the formation of proteoliposomes.
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Figure 0-7: Western blots of OR subunits reconstituted into liposomes. A) His_{10}^\text{Myc}-DmOrco and B) His_{10}^\text{Flag}-DmOr43b were reconstituted into preformed liposomes together. Lane numbers 1-8 indicate fractions taken from the bottom of the tube after density gradient ultracentrifugation, lane 1 indicates the bottom of the gradient and lane 8 indicates the top. Both subunits were integrated into liposomes that float to fraction 7 at the top of the tube. A) anti-myc western, B) anti-flag western.

The different proteoliposome preparations were treated with each of the four cross-linking reagents at a concentration of 5 mM (Figure 0-8). The BMPA reagent did not have any effect on the banding pattern of the proteins when compared to the untreated sample (Figure 0-8 lanes marked 4). However, when the proteoliposomes were treated with DSS, DSG or BS3 there were no bands evident on the gel (Figure 0-8 lanes marked 1-3), possibly due to the formation of large cross-linked aggregates that could not enter the gel matrix.

Figure 0-8: SDS-PAGE analysis of OR subunits reconstituted into liposomes and cross-linked with 5 mM of each reagent. Purified His_{10}^\text{Flag}-DmOr43b and His_{10}^\text{Myc}-DmOrco and were reconstituted into pre-formed liposomes either separately A) His_{10}^\text{Flag}-DmOr43b and C) His_{10}^\text{Myc}-DmOrco, or together B), and incubated with 5 mM of each cross-linking reagent, U) untreated, 1) DSS, 2) DSG, 3) BS3, 4) BMPA.
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There is evidence of the presence of higher order bands for each of the proteoliposome preparations in SDS PAGE analysis (Figure 0-9). The banding pattern for the DmOrco subunit in liposomes (Figure 0-9 C) looks very similar to that of the purified subunit (Figure 0-6). This banding pattern is modified when both subunits are reconstituted in the same liposome preparation (Figure 0-9 B), and is different from the bands observed for the DmOR43b subunit alone (Figure 0-9 A). This modification in banding pattern could indicate that the two subunits are interacting in the liposome.

I then tested whether reducing the concentration of each cross-linker would have a similar affect on the proteoliposomes by treating them with three lower concentrations; 0.1 mM, 0.01 mM and 0.001 mM. There was no change in the banding pattern on SDS PAGE (Figure 0-9) when the liposomes were incubated with BMPA at all concentrations or with the other three cross-linking reagents at either 0.001 mM or 0.01 mM concentrations, compared to the untreated samples (Figure 0-9 lane U). However when DSS, DSG and BS3 were added at 0.1 mM the protein bands were either greatly reduced or not evident on the gel at all, this could be due to the cross-linking reagent creating protein aggregates that are too large to enter the gel matrix (Figure 0-9 A, B and C, lanes marked a).
Figure 0-9: SDS PAGE analysis of purified OR subunits integrated into pre-formed liposomes after treatment with cross-linking reagents. Purified His\textsubscript{10}-Flag-DmOr43b and His\textsubscript{10}-Myc-DmOrco were inserted into pre-formed liposomes either separately A) His\textsubscript{10}-Flag-DmOr43b and C) His\textsubscript{10}-Myc-DmOrco, or together B), and incubated with three concentrations of each cross-linking reagent, a) 0.1 mM, b) 0.01 mM, or c) 0.001 mM. U) indicates the untreated sample.
3.3.3 Size Exclusion Chromatography – Multi Angle Light Scattering (SEC-MALS)

3.3.3.1 SEC-MALS analysis of insect OR subunits in Zwittergent 3-16

In order to determine the molecular weights of the potential homomeric structures for each of the OR subunits, SEC-MALS analysis was carried out on each subunit in solution.

The His\textsubscript{10}-Flag-DmOrco subunit was injected onto the gel filtration column in 100 µL aliquots at three different concentrations, 0.8 mg/mL, 0.4 mg/mL and 0.2 mg/mL. The samples were run at 0.5 mL/min for 50 min. Each of the three SEC-MALS runs gave very similar profiles for all three detectors (data not shown). These profiles indicate that in the conditions used DmOrco is predominantly forming a molecular weight of ~55 kDa (Figure 0-10 E), with a small proportion at ~120 kDa (Figure 0-10 E).

The His\textsubscript{10}-Myc-DmOrX subunits in buffer containing 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, 0.2 mM Zwittergent 3-16, were injected in 100 µL aliquots at the following concentrations; 1.5 mg/mL and 0.5 mg/mL (DmOr10a), 2.8 mg/mL and 1.5 mg/mL (DmOr22a), 2.6 mg/mL and 0.8 mg/mL (DmOr43b), and 2.3 mg/mL and 0.7 mg/mL (DmOr67d). The profiles from the two different injections were very similar for each of the OrX subunits (data not shown); however, the diluted sample generally resulted in less aggregation and a better analysis of the MW. Like DmOrco, three of the receptors; DmOr10a, DmOr22a ad DmOr67d had a dominant peak with a small shoulder (Figure 0-10 A, B and D). The DmOr43b sample produced two UV peaks (P1 and P2) on the SEC profile when it was purified (Figure 0-3 C), these two peaks were collected and concentrated and analysed separately by SEC-MALS. The resulting profiles for the two samples were identical (data not shown). The SEC-MALS data indicate that there is one dominant UV peak with a small shoulder (Figure 0-10 C purple line); however the MALS data only indicates the presence of ordered protein at the larger peak.
3.3.3.2 SEC-MALS analysis of DmOrco exchanged into other detergents

To examine the potential for OR subunits to form oligomers in other detergents, the purified DmOrco subunit was exchanged into three other detergents on a gel filtration column (Figure 0-11). The protein maintained a homogenous peak in both n-Dodecyl β-D-Maltopyranoside (DDM) and Brij 35 (Figure 0-11 A and B), however when exchanged into sodium cholate it formed a large aggregate at the void volume for the column (Figure 0-11 C).
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Figure 0-11: SEC profiles for DmOrco when exchanged into three new detergents. DmOrco was solubilised using Zwittergent 3-16 and exchanged into; A) Brij 35, B) DDM or C) sodium cholate on the gel filtration column. The blue line indicates the DmOrco protein, the dotted line indicates standards with peaks from left to right corresponding to; 444 kDa, 200 kDa, 66 kDa, 29 kDa and 13.7 kDa.

The DmOrco subunit in buffer containing 50 mM NaH₂PO₄, 150 mM NaCl, with either 0.5 mM DDM or 0.3 mM Brij 35 was injected on to the SEC-MALS at 2 mg/mL (Figure 0-12 A and B). There was a large amount of aggregated protein in the DDM sample (Figure 0-12 A) as indicated by a large peak at the estimated void volume (Figure 0-12 A).

When DmOrco was exchanged into Brij 35 there was very little aggregated protein evident as there was no indication of a peak at the void volume, there were however two UV peaks both of which contained a monomeric DmOrco (Figure 0-12 B). This may be due to differing amounts of detergent surrounding the protein at these particular elution volumes.
3.3.4 Surface Plasmon Resonance analysis of interactions between DmOrco and DmOr22a

In order to carry out interaction studies between DmOr22a and DmOrco using SPR, I first needed to determine the optimal immobilisation approach to couple OR subunits to the surface of the sensor chip. The first SPR experiment was designed to immobilise the OR subunits that had been expressed in the wheat germ cell-free system by exploiting the His\textsubscript{10}-tag to bind these receptors to the surface of Ni-NTA sensor chip. However, this resulted in a large amount of non-specific binding of proteins present in the wheat germ reaction mix. Figure 0-13 shows that when Myc-DmOr22a or a control wheat germ cell-free reaction without an expressed protein, was passed over the Ni-NTA surface, they produced a response profile similar to that of the His\textsubscript{10}-DmOr22a. This would imply that either there are proteins in the reaction mix that are binding to the chip surface at a level higher than that of...
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the His\textsubscript{10}-tagged OR subunit, or the His\textsubscript{10}-tag is not binding to the Ni-NTA chip surface. However, the wheat germ cell-free kit that we are using (WEPRO7240H) has been processed by the manufacturer to remove endogenous proteins that non-specifically bind to His-affinity resin.

![Figure 0-13: SPR sensorgram demonstrating the non-specific binding of proteins in the wheat germ cell-free reaction. The red trace is from a His\textsubscript{10}-DmOr22a reaction, the green trace is Myc-DmOr22a and the black trace is an empty wheat germ cell-free reaction. RU – arbitrary response units.](image)

The experiments were then modified to determine if the other epitope tags could be used to specifically bind the OR subunits expressed in wheat germ cell-free reactions. An anti-Myc antibody was covalently bound to the surface of a CM5 chip to enable immobilisation of the Myc-DmOr22a subunit. The immobilisation of antibody to the surface was indicated by an increase of 12,000 RU compared to the baseline (Figure 0-14). The Myc-DmOr22a receptor was then immobilised on the myc antibody that had been covalently bound to the chip surface (Figure 0-15 injection 1), indicated by the increase in binding that stabilised at 150 RU. A second injection of Or22a was carried out to improve stability of the baseline (Figure 0-15 injection 2). Some leaching of protein was evident through the steady decline in RU, estimated to be approximately 7 RU/min. Injecting Flag-DmOrco resulted in an increase in RU of 100 (Figure 0-15 injection 3), indicating a potential interaction between these two receptors. Injecting Flag-DmOrco or a control wheat germ reaction mix directly onto the
bound anti-Myc antibody did not result in an increase in RU indicating that the change in mass was not due to non-specific binding of proteins to the antibody (data not shown).

Figure 0-14: Immobilisation of Myc antibody to the CM5 chip surface through amine coupling. Numbers indicate 1) activation of the chip surface, 2) injection of Myc antibody, 3) blocking of the chip surface with ethanolamine.

Figure 0-15: SPR sensorgram investigating interactions between DmOr22a and DmOrco. Numbers 1 – 3 indicate the following injections; 1 – Myc-DmOr22a, 2 – Myc-DmOr22a, 3 – Flag-DmOrco.

To ensure the reliability of SPR experiments, and determine the binding kinetics, it is necessary to be able to repeat the immobilisation procedure. The chip surface therefore, has to be regenerated and a similar level of protein immobilised again under the exact same conditions. I found that it was not possible to regenerate the chip surface when the OR
subunit was immobilised on a covalently bound primary antibody. Several regeneration strategies were attempted without success including; 0.1 M glycine at pH 2-5; 0.1 M triethanolamine pH 11.5; Formic acid pH 2; 50 mM NaOH; 3 M MgCl; and 25-50% ethylene glycol (data not shown). After consultation with BIAcore representatives I then decided to covalently bind a secondary antibody to the chip surface and immobilise the primary anti-Myc antibody to this. Myc-tagged protein could then be immobilised on the primary antibody. It should then be feasible to regenerate the interaction between the two antibodies allowing repetition of experiments. Using this approach a primary antibody could be immobilized on a secondary antibody that was covalently bound to the chip surface (Figure 0-16 A). The DmOrco subunit was then immobilized to the primary antibody (Figure 0-16 B), and the chip surface was subsequently regenerated to baseline levels (Figure 0-16 C). However at this stage the BIAcore machine that I was using broke down and was not repaired in sufficient time to finish the experiments.

![Figure 0-16](image.png)

Figure 0-16: SPR sensorgram showing successful regeneration of chip surface. Anti-Flag antibody was covalently bound to the chip surface and the baseline was set at zero. (A) Primary antibody (anti-Flag) was injected. (B) His10-Flag-DmOrco was injected. (C) 10 mM glycine pH 2 was injected to regenerate the chip surface back to baseline levels indicated by the dashed line.
3.4 Discussion

This chapter tested the hypothesis that insect OR subunits can form homo- and heteromeric complexes. Four techniques were used to investigate this hypothesis; Native PAGE gels, chemical cross-linking, SEC-MALS and SPR.

The Native Page gels provided evidence that the subunits are capable of forming higher order structures in a gel matrix. However, this was detergent dependent with some detergents causing the protein to aggregate or otherwise inhibit migration through the gel, resulting in smears and large blobs of indiscernible size. The DmOrco subunit was the most stable subunit in the Native Gels and showed a distinct pattern of oligomeric structure indicating potential dimers, trimers, tetramers, and hexadecamers (16-mers). The mobility of the OrX subunits varied between the four receptors tested. The formation of discrete bands for all OrX subunits was only observed after addition of either 4% PFO or 4% SDS, resulting in potential monomers, dimers, trimers, tetramers, and 16-mers. The OrX subunits were unstable at room temperature and prone to precipitating out of solution in the buffer conditions chosen for purification (50 mM NaH$_2$PO$_4$, 150 mM NaCl, 0.2 mM Zwittergent 3-16). This might account for the level of aggregation observed for this condition on the gels. The addition of 4% Zwittergent 3-16, 4% DDM or 4% Brij 35 also resulted in a smear on the gel for all the receptors tested. This might be due to the protein aggregating under these conditions, or these detergents might be inhibiting the ability of the Coomassie dye to impose a charge on the protein-detergent complexes and thus preventing them from migrating through the gel matrix (Wittig et al. 2006).

The detergent perfluorooctanoate (PFO) has been used to enhance the ability of membrane proteins to migrate through Native Gels as it does not break the protein:protein bonds (Ramjeesingh et al. 1999). This detergent was trialled and found to produce a similar banding pattern as that seen with SDS. It is interesting to note that there was evidence of oligomers forming within the gel even in an excess of the denaturing detergent SDS (Figure 0-5). This correlates with the appearance of similar higher-order bands in SDS PAGE analysis of the purified subunits (Figure 0-6).
To further investigate the oligomeric structure of DmOrco, the purified protein was incubated with four different cross-linking reagents in an attempt to ‘fix’ the protein in one or more oligomeric states. However, the addition of the crosslinking reagents did not have an observable effect on the higher order structure of this subunit using SDS-PAGE. This might be due to the detergent micelle environment that the receptor has been purified and stored in. The detergent may envelope the receptor in its monomeric state in such a way as to restrict the availability of the amino acids required by each reagent for cross-linking. The OrX subunits were unstable at room temperature and so could not be incubated with the cross-linking reagents for the length of time required for this experiment. If conditions are found to increase the stability of these subunits, these cross-linking reactions could be carried out to investigate the potential of ‘fixing’ the higher order complexes.

One way to circumvent these problems posed by the addition of detergent is to reconstitute the protein into liposomes and then carry out the crosslinking reaction. In the liposome reconstitution procedure the excess detergent is removed and thus will not have an effect on the cross-linking reactions. There is also a greater chance that the protein will fold and associate correctly when reconstituted into an artificial lipid membrane. However, there was no evidence of the formation of oligomers with any of the cross-linking reagents used in this study even though two of the reagents (DSS and DSG) are membrane permeable. This might be due to the choice of cross-linking reagent and perhaps another reagent that was not tested might potentially crosslink the proteins. The manufacturer of the cross-linking reagents used here (Proteochem) list over 30 additional cross-linking reagents that could be tested. However, it is more likely that the subunits are not interacting to form a complex when incorporated into liposomes. It would be beneficial to have a functional assay for the proteoliposomes to ensure they are folding correctly and interacting. We have begun to investigate the possibility of using a Planar Lipid Bilayer system (Dalziel et al. 2007) to monitor the activation of insect ORs with their respective ligands. However, this work is only at a preliminary stage and no definitive results have been obtained as yet.

The protein loaded onto the Native Page and SDS PAGE gels is in a detergent solubilised form and must therefore be considered a protein-detergent complex. Thus, the MW of the bands that can be visualized on the gels may not necessarily relate to the size of the protein.
oligomers, as a portion of the mass can be attributed to the detergent. To differentiate between the protein-detergent complex and the protein alone, the subunits were analysed with SEC-MALS, which has the ability to subtract the mass of the detergent from the complex.

The SEC-MALS analysis indicated that in the presence of 0.2 mM Zwittergent 3-16, each of the OR subunits are predominantly monomeric, with some dimer formation evident for DmOr10a, DmOr22a, DmOr67d and DmOrco. The elution volume of the DmOrco subunit changed depending on which detergent was in the buffer (Figure 0-10 E, and Figure 0-12 A and B). This is an example of why elution volumes from standard SEC profiles should not be used to determine the MW of membrane proteins in detergent buffers. The size of the monomers and dimers formed for each of the OrX subunits was slightly smaller than that expected, with the estimated MW of these proteins between 48 kDa and 50.4 kDa. The SEC-MALS analyses indicated a MW of 40 to 44 kDa for the monomers and between 75 and 90 kDa for the dimers. This error in estimation could be due to the level of aggregation in these samples affecting the light scattering through the analysis. There is a long sloping shoulder on each of the traces from close to the void volume through to the formation of the first definitive peak (Figure 0-10). This is an indication of the presence of aggregated protein in the sample. As mentioned earlier the DmOrX subunits were not very stable at room temperature and it is possible that the protein aggregated either prior to injection or at some stage during the analysis. The protein that remained in the tube after injection precipitated before the analysis was complete indicating the instability of these receptors in solution.

To examine the potential of insect ORs to behave differently in different detergents, DmOrco was exchanged into three other detergents. Its oligomeric state was modified in 0.5 mM DDM with predominantly dimers being formed. However, DmOrco was prone to aggregation in this detergent, which has an effect on the MW analysis (David Goldstone pers. comm.), resulting in a shift of the MW values which do not correspond to the same elution volume as the UV and RI traces This resulted in MALS spectra that were difficult to analyse and this result may be an artifact of the aggregate rather than a true representation of the multimeric state of DmOrco.
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This SEC-MALS analysis also provided evidence that the DmOrco subunit is inherently more stable than the other ORs and is less prone to aggregation. The DmOrX subunits tended to precipitate out of solution if left at room temperature for longer than 10-20 minutes. If they are maintained at 4°C the detergent precipitates resulting in the protein precipitating again. The OR subunits might be more stable when exchanged into other detergents, however this was not tested. Further investigation is needed to find the optimum storage conditions for these receptors. The DmOrco subunit however, could be stored at room temperature for days without precipitating, and thus was much more amenable to analysis. It is likely that the OR subunits require the presence of Orco to enhance their stability. It has been shown that both in vivo and in heterologous cells the stability of the subunits is increased in the presence of Orco (Larsson et al. 2004; Nakagawa et al. 2005; Neuhaus et al. 2005; Benton et al. 2006).

The SPR experiments provided evidence that an interaction between the insect OR subunits does occur. Immobilised DmOr22a appears to interact with DmOrco under the conditions trialed. However, as the chip surface could not be regenerated this experiment could not be replicated. There are also a number of control experiments that need to be carried out to ensure the validity of this result, such as the addition of a non-OR protein, or an OR from a different insect species. However, if this result holds with controls it opens the door to an entirely new way of investigating interactions between the insect OR subunits. It will also allow for investigation of ligand binding with the formation of the OR complex in artificial membranes on the chip surface.

The immobilisation strategies tested in this study could be expanded to aid in the regeneration of the chip surface. Membrane proteins can be covalently bound directly to the chip surface using standard amine coupling chemistries (Karlsson and Lofas 2002; Arnold and Fremont 2006; de Kloet et al. 2010; Pixley et al. 2011). However, this immobilisation technique fixes the protein in a very specific state and does not allow the protein freedom to move. It is also completely removed from a lipid environment and thus might not be beneficial for studying membrane protein interactions. Two approaches have been used to form lipid bilayers on the sensor surface: through the deposition of lipids containing protein directly on the chip (Puu and Gustafson 1997; Knoll et al. 2000), which has proven useful to
support the activity of some membrane proteins (Puu and Gustafson 1997), and depositing a layer of lipids onto a previously attached hydrophobic monolayer (Lang et al. 1994; Cooper et al. 1998) then binding the membrane protein to this. BIAcore produce a HPA chip for their machines which has a monolayer of long-chain alkanethiol groups covalently attached to the gold surface. Although the hydrophobic layer can be useful for embedding membrane proteins, it can be problematic for transmembrane proteins as the internal loops will be in contact with the solid gold surface which may inhibit correct folding and functionality. To lift the bilayer from the surface and allow the internal loops space to fold correctly, the bilayer can be tethered to hydrogels (soft polymer cushions) (Sackmann 1996) with various lengths of linkers. There are also novel methods for immobilising the protein such as the use of nanodiscs which have been used to screen lipid-protein interactions, and protein-protein interactions, and the production of nanosomes directly from cell membranes that will allow the protein to remain in a native environment (Vidic et al. 2006; Benilova et al. 2008).

This chapter set out to investigate the potential of insect OR subunits to form homo- and heteromeric complexes. The oligomeric state of proteins in solution is dependent on the conditions used to purify them and the storage and analysis buffers. The buffer conditions chosen for the biochemistry analyses carried out in this study resulted in the purification of receptors in a predominantly monomeric form with the potential to form higher order complexes. It would appear that these receptors are capable of forming oligomers but the process is likely to be in equilibrium with a constant flux between states. This is evident from the DmOR43b subunit that formed two peaks when analysed by SEC, indicating this receptor was potentially forming two oligomeric states with different molecular weights. However, when the two peaks were analysed separately by SEC-MALS and Native PAGE they provided evidence that both peaks were forming similar oligomeric structures, which would suggest the two states observed with SEC are not stable but rather in equilibrium. The oligomeric state also appears to be more stable in a gel environment as both SDS PAGE and Native PAGE indicate the presence of higher order species, though the SEC-MALS analysis limits this to predominantly monomers with a small amount of dimer.

To increase the probability of producing oligomeric structures a further detergent screen should be carried out on the purified receptors. This can be carried out using a Ni-NTA
column to bind the His\textsubscript{10}-tagged receptors and eluting them in buffer containing the different detergents to be screened. The receptors can then be examined for oligomeric structures using SEC-MALS. I have demonstrated that the receptor complexes are not very stable under the conditions used in this study. There are a variety of stabilizing compounds that could be added to the purification procedure to increase the stability of these receptor complexes, although it is likely that a different detergent will also have some stabilizing effect. These stabilizing compounds can include natural ligands or binding partners, perhaps purifying both His\textsubscript{10}-DmOrco and a His\textsubscript{10}-DmOrX together will enhance the stability of the OrX subunit. There are a number of natural ligands for the OrX subunits that could be trialed, as well as the VUAA1 molecule that has been shown to activate DmOrco. It has also been demonstrated that glycine and proline (Wang et al. 2013), as well as glycerol, sucrose, dimethylsulfoxide, mannitol, inositol, and xylitol (Fan et al. 2012), can be used to reduce aggregation and increase stability of proteins during purification. The addition of reducing agents can prevent aggregation through the reduction of disulphide bonds between cysteine residues, and number of different reducing agents can be trialed, including DTT, β-mercaptoethanol and tris(2-carboxyethyl)phosphine (TCEP).

This study is the first to investigate the higher order structure of the insect ORs. Although previous reports (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013) have determined a potential interaction between the OR subunits none have gone on to investigate either the stoichiometry of the subunits involved, or the size of the potential oligomers formed. However, the results obtained in this study are not conclusive enough to draw any inferences about the oligomeric state of these receptors \textit{in vivo}, and further work is needed to assess the oligomeric state of both the individual OR subunits and the potential OR complex.
Further Investigation of Interactions between Insect Odorant Receptor Subunits
**4.1 Introduction**

Evidence from the literature suggests there is likely to be an interaction between Orco and OrX to ensure correct membrane trafficking and to confer a response upon ligand binding (see Section 3.1). In *Drosophila melanogaster*, Orco is thought to interact with all 61 ligand-binding ORs independently, yet thorough analysis of the sequences of these ligand-binding ORs has not revealed a common binding site for this interaction.

**4.1.1 Interaction Studies**

Protein complementation assays using a split yellow fluorescent protein have shown that a ligand binding OR (DmelOr43a) interacts with itself and with Orco (Benton et al. 2006), however, this assay does not offer any insight into where the binding occurs between the receptors. Yeast 2 hybrid experiments indicate that an interaction is occurring between intracellular loop three of the ligand binding OR and intracellular loop three of Orco (Benton et al. 2006). This is the only study to date that has identified an interaction site between the ORs. Resonance Energy Transfer (FRET) experiments have shown that ORs can form both homomeric and heteromeric interactions in heterologous cell systems (Neuhaus et al. 2005; German et al. 2013). However, there is no data on the functionality or stoichiometry of these complexes, or the site of the interaction.

Although there have been a number of studies implying a physical interaction between Orco and OrX, these have been predominantly carried out in heterologous cell systems (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013), with only one group showing a biochemical interaction outside of a cell (Tsitoura et al. 2010). Mosquito ORs have been demonstrated to interact with Orco expressed in a moth cell line using co-immunoprecipitation (pull-down assay).

There are inherent problems when taking a classic protein biochemistry approach to investigating interactions between membrane proteins. Firstly there is no functional assay for the purified insect ORs to ensure that the proteins have folded and are interacting correctly. Secondly, the choice of buffer conditions and detergents can have an impact on the behaviour of the proteins in solution, and thirdly even if all the conditions are met and
the protein is folding correctly and forming a functional complex there is the question of whether it is behaving as it would in vivo.

To overcome some of these problems, in this chapter I will be looking at the OR complex in cell membranes. Although this is not a substitute for in vivo studies, insect ORs are known to be functional in HEK 293 cells (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008; Jones et al. 2011; Sargsyan et al. 2011; Jones et al. 2012) and have been shown to interact when expressed in insect cells (German et al. 2013).

4.1.2 Methods for Investigating Interactions

4.1.2.1 Pull down assays

Co-immunoprecipitation or pull down assays are a tool that is used to investigate protein-protein interactions, particularly when the proteins form subunits of a larger complex. Pull down assays utilise an antibody that recognises an epitope or epitope tag on a known protein. If this protein is part of a larger complex then the other subunits may be pulled down with the known protein when it is bound to an antibody that is immobilised on a solid surface such as agarose. This technique has been used to demonstrate an interaction between mosquito ORs expressed in a Bombyx mori cell line (Tsitoura et al. 2010), as well as other membrane proteins in heterologous cells (Ho et al. 2012; Basit et al. 2013; Montesinos et al. 2013).

4.1.2.2 Crosslinking

The use of the cross-linking reagents as outlined in section 3.1.3.2 will allow an analysis of the interactions between subunits should they prove to be transient or weak in nature. This strategy has been used to study membrane proteins in heterologous cells (le Maire et al. 2007; Krupnik et al. 2011; Huysmans et al. 2012).

4.1.2.3 Single Molecule Fluorescence Photobleaching

To investigate the homo- or heteromeric structure of the insect ORs without the need to solubilise in detergents, a single molecule photobleaching techniques was utilised. Tagging membrane proteins with a GFP molecule allows the oligomeric structures to be visualised as
a single molecule. Under continuous excitation at low intensity the fluorescence emitted by the GFP molecules decreases in approximately equal sized steps consistent with the bleaching of single molecules (Ulbrich and Isacoff 2007; Abuin et al. 2011; Coffman and Wu 2012; Hallworth and Nichols 2012; Leake 2013). The number of subunits in a complex can be deduced by counting the number of steps required to bleach the molecules to background fluorescence levels (Figure 4-1). The appropriate light intensity and exposure time differ depending on the number of fluorescent molecules in the protein complex of interest and this will have to be deduced empirically.

![Figure 4-1: Single molecule fluorescence photobleaching trace of the Drosophila melanogaster calcium selective ion channel (Orai1). eGFP bleaching steps are indicated by arrows, two steps indicate this molecule is a dimer. Modified from Demuro et al. (2011).](image)

This technique has provided insights into the subunit stoichiometry of neurotransmitter receptors (Durisic et al. 2012; McGuire et al. 2012), Ionotropic Glutamate receptors (Abuin et al. 2011) and motor proteins such as prestin (Hallworth and Nichols 2012) and MotB (Leake et al. 2006). Using GFP alone allows analysis of the stoichiometry of the homomeric subunits, while the use of two fluorescent proteins (GFP and mCherry) can allow analysis of the stoichiometry of the subunits in the complex by examining the molecules where the fluorescence intensities of the two fluorophores overlap (Benton et al. 2006).

### 4.1.3 Aims

I have demonstrated in Chapter 3 that the insect ORs are capable of forming higher order structures using Native PAGE gels and SEC-MALS, and a heteromeric interaction could occur between an immobilised DmOr22a subunit and DmOrco using SPR (section 3.3.4). In this
Chapter this interaction will be further investigated using pull-down assays, cross-linking experiments and single molecule fluorescence photobleaching.

In this chapter I will use three new approaches to investigate the potential interaction.

1) Pull down assays

Receptor subunits expressed in HEK 293 cells are known to be functional. If the subunits from a functional complex in the cell membrane when expressed together, or stable homomers when expressed alone, tagging one of the subunits with a His$_{10}$ epitope should allow the purification of both subunits on a Ni-NTA column.

Receptor subunits will also be expressed using the baculovirus-mediated Sf9 expression system as this produces large amounts of protein that are amenable to solubilisation and purification (Chapter 3), and it has been demonstrated that the OR subunits interact in this system (German et al. 2013). However, unlike the HEK 293 system, there is no functional assay for insect ORs expressed using baculovirus in Sf9 cells as the viral infection prevents the cells from adhering to assay plates.

The wheat germ cell-free expression system will also be trialled in these experiments as the literature has shown that this system contains plant lipids that could be acting as membranes, and mammalian ORs can be expressed, purified and used in ligand-binding assays. However, there is no functional assay for the insect ORs in this system.

2) Chemical Cross-linking

If there is no evidence of an interaction using the pull-down assay it might be due to the complex formation being transient or unstable. To address this issue five cross-linking reagents will be examined for their ability to covalently ‘fix’ subunits in their oligomeric state to enable co-immunoprecipitation.

3) Single Molecule Fluorescence Photobleaching

It has been shown using FRET that insect ORs interact in heterologous cells when tagged with fluorescent proteins (German et al. 2013). To analyse the oligomeric state of the
complex in cell membranes I will use single molecule fluorescent photobleaching. The subunits will be expressed with a GFP tag in HEK 293 cells, as this is the only system that has provided us with functional data for these receptors, and has been used in the literature to perform this type of experiment on membrane protein complexes (Hallworth and Nichols 2012). The GFP molecule should bleach in a stepwise manner when subjected to a continuous low level of fluorescent light. The number of steps required to bleach the GFP molecule to background levels will indicate the number of molecules in the protein complex.
4.2 Methods

Six odorant receptors from *D. melanogaster* were used in this study; DmOrco, DmOr10a, DmOr22a, DmOr35a, DmOr43b and DmOR67d. Each of the receptors was tagged at the N terminus with a His<sub>10</sub>, Flag, Myc, His<sub>10</sub>-Flag, and a His<sub>10</sub>-Myc epitope tag. The design and production of the constructs is outlined in section 2.2.2.

The insect OR subunits were expressed in three different eukaryotic expression systems; a wheat germ cell-free system, baculovirus-mediated expression in Sf9 cells, and an inducible HEK-293 cell system. Two interaction experiments were carried out. Firstly the subunits were co-expressed to examine whether they formed a complex either in the cell membranes or in the cell-free reaction. Secondly the subunits were expressed individually to determine whether they would interact with a subunit that was immobilised on Ni-NTA resin.

4.2.1 Expression of insect OR subunits

4.2.1.1 Baculovirus mediated expression in Sf9 cells

Sf9 cells were maintained in Sf900II media (Life Technologies) at 27°C with shaking at 100 rpm. For co-expression experiments, 180 mL at 2 x 10<sup>6</sup> mL<sup>-1</sup> were infected with baculovirus at an MOI of 1 for each virus, for separate expression experiments two flasks containing 90 mL of Sf9 cells at 2 x 10<sup>6</sup> mL<sup>-1</sup> were infected with baculovirus containing either the His<sub>10</sub> tagged subunit or the non-His tagged subunit at an MOI of 1 for each virus. Cells were incubated at 27°C for 72 hours with shaking at 100 rpm. The cell pellets were collected by centrifugation at 3800g for 10 min at room temperature and then resuspended in 20 mL each of resuspension buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.5, 150 mM NaCl, 1x protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)), with 25 U/mL Benzonase, then lysed by two passes on an Emulsiflex C5 emulsifier (Avestin, Germany) at 10,000-15,000 psi. The sample was then centrifuged at 1000 g for 5 min to remove whole cells and nuclei. The supernatant was removed and spun at 100,000 g for 1 hour at 18°C. The membrane pellet was resuspended in 6 mL of buffer A, with 10 mM MgCl<sub>2</sub> and 1% w/v detergent (Zwittergent 3-
16) and rotated for 1 hour at room temperature at 10 rpm. The sample was then centrifuged at 100,000 g for 1 hour at 18°C.

4.2.1.2 Expression of insect OR subunits in HEK 293 cells

4.2.1.2.1 Cloning and Stable Cell Line production

His$_{10}$-Flag-OR67d, His$_{10}$-Flag-OR10a and His$_{10}$-Flag-OR10a were gateway cloned into the TRex-pDest-30 vector (Life Technologies). The constructs were linearised using the MfeI restriction enzyme (New England Biolabs), gel purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and transfected into a Flp-In™ 293 T-Rex cell line stably expressing Myc-DmOrco using Lipofectamine 2000 (Life Technologies) as per the manufacturer’s instructions. Cells were grown to 80% confluency in T25 flasks with Dulbecco’s Modified Eagle Media (DMEM (Life Technologies)) then placed under selection using 500 µg/mL G418. Cells were again grown to confluency and passaged three times after which aliquots were frozen at -80°C. Prior to use in experiments, cells were defrosted and cultured for three passages under selection.

For co-expression experiments, 6x10$^7$ cells expressing both Myc-DmOrco and the His$_{10}$-Flag-OR were harvested, for separate expression experiments 6x10$^7$ cells expressing Myc-DmOrco and 6x10$^7$ cells expressing the His$_{10}$-Flag-OR were harvested. The cells were pelleted by centrifugation at 3800g for 10 minutes at room temperature and then resuspended in 2 mL of resuspension buffer A, with 25 U/mL Benzonase, then lysed by sonicating three times at 20% power for 10 seconds with a 1 minute incubation on ice between each sonication step. The sample was then centrifuged at 1000g for 5 minute to remove whole cells and nuclei. The supernatant was removed and spun at 100,000g for 1 hour at 18°C. The membrane pellet was resuspended in 1 mL of buffer A, with 10 mM MgCl$_2$ and 1% w/v detergent (Zwittergent 3-16) and rotated for 1 hour at room temperature at 10 rpm. The minimum volume for the centrifuge tubes is 3 mL so 2 mL of buffer A with 10 mM MgCl$_2$ and 1% w/v Zwittergent 3-16 was added to the sample prior to centrifugation at 100,000 g for 1 hour at 18°C.
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4.2.1.3 **Expression of insect OR subunits in the wheat germ cell-free system**

Wheat germ cell-free reactions were performed as described in section 2.2.4.2. The completed translation reactions were pooled and centrifuged using an airfuge (Beckman Coulter) at 30 psi for 1 hour (> 100,000 g). The pellet was resuspended in 1 mL of resuspension buffer A with 1% w/v Zwittergent 3-16 and rotated for 1 hour at room temperature at 10 rpm. The sample was centrifuged in an airfuge at 30 psi for 1 hour, and the supernatant was removed.

4.2.2 **Pull down assay protocols**

4.2.2.1 **Pull down assay protocol for co-expression experiments in Sf9 or HEK 293 cells**

The supernatant was removed and loaded onto a 1 mL Ni-NTA column (GE Healthcare). The column was washed in ten column volumes of buffer B (50 mM NaH$_2$PO$_4$ pH 7.5, 0.2 mM Zwittergent 3-16) with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes buffer B with 150 mM NaCl and 250 mM imidazole. An interaction between the subunits was evaluated by loading equal volumes of each sample on SDS PAGE gels and analysing the bands by western blot using antibodies against the epitope tags on each protein (Flag or Myc) as described in section 2.2.5.

4.2.2.2 **Pull down assay protocol for separate expression experiments in Sf9 or HEK 293 cells**

The supernatant from the His$_{10}$ tagged infected cells was loaded onto a 1 mL Ni-NTA column (GE Healthcare). The column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. The supernatant from the non-His tagged subunit was then loaded onto the Ni-NTA column. The column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes buffer B with
150 mM NaCl and 250 mM imidazole. An interaction between the subunits was evaluated by western blot against the epitope tag on each protein as described in section 2.2.5.

**4.2.2.3 Pull down assay protocol for OR subunits co-expressed in the wheat germ cell-free system**

The wheat germ cell-free reaction is limited to 10 µL of RNA which is the standard size of the reaction for the WEPRO kit (CellFree Sciences). To increase the amount of protein translated when two different receptors were expressed in the same reaction, I made one standard RNA reaction for each subunit as per the manufacturer’s instructions. This RNA was then purified using a phenol chloroform method and resuspended in half the original volume using nuclease free water. This extraction resulted in 5 µL of each RNA sample, and a total of 10 µL of RNA for co-expression studies. For each pull-down experiment two standard wheat germ cell-free reactions were used.

The solubilised OR subunits were mixed with 200 µL of Ni-NTA slurry (Qiagen) that had been pre-washed with buffer A containing 1% w/v detergent (Zwittergent 3-16). The sample was rotated for 1 hour then packed into a column, the flow-through was collected and the column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes of buffer B with 150 mM NaCl and 250 mM imidazole. An interaction between the subunits was evaluated by western blot against the epitope tag on each protein as described in section 2.2.5.

**4.2.2.4 Pull down assay protocol for OR subunits separately expressed in the wheat germ cell-free system**

For each pull down experiment two standard reactions of each subunit were produced and pooled. The supernatant from the His$_{10}$-tagged subunit was mixed with 200 µL of Ni-NTA slurry (Qiagen) that had been pre-washed with buffer A containing 1% w/v detergent (Zwittergent 3-16). The sample was rotated for 1 hour then packed into a column, the flow-through was collected and the column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 250 mM imidazole, and a further ten column volumes of buffer B with 150
mM NaCl and 50 mM imidazole. The supernatant from the non-His_{10}-tagged protein was loaded on the column and the flow-through was collected. The column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes of buffer B with 150 mM NaCl and 250 mM imidazole. An interaction between the subunits was evaluated by western blot against the epitope tag on each protein as described in section 2.2.5.

I also tested whether mixing the two completed wheat germ reactions together prior to loading on the Ni-NTA column would facilitate an interaction between the subunits. In this case the two subunits (one His_{10}-tagged and the other not) were allowed to mix on a rotator for 30 minutes. The sample was treated as in section 4.2.2.3.

### 4.2.3 Calcium based influx assay for HEK 293 expressed OR subunits

Each cell line of interest was plated into black-walled, poly-d-lysine coated, 96-well plates (BD Biocoat) at 25 000 cells/well in 100 µL DMEM (Life Technologies) and incubated for ~24 hrs at 37°C with 5% CO₂. The media was removed and the top four rows of wells were replenished with fresh DMEM. The bottom four rows of wells were replenished with fresh DMEM containing 1 µg/mL doxycycline (SigmaAldrich) to induce protein expression. The cells were incubated for 16 hrs at 37°C with 5% CO₂. The media was removed and the cells were washed once with assay buffer (PBS + 1 mM Probenecid, pH7.4), then replaced with 50 µL of assay buffer containing 1 µM Fluo-4 (Life technologies) + 0.02% pluronic acid. The cells were incubated for 30 minutes at room temperature in the dark. The loading solution was removed and the cells were washed twice with 100 µL of assay buffer, 100 µL of assay buffer was added to the wells and the plate was incubated for 30 minutes at room temperature in the dark. The assay was carried out on a Fluostar Plate Reader (BMG Labtech). Fluorescence was measured with excitation at 488nM and emission at 520nM. The fluorescence was measured once for each of the 6 wells assayed to give a background level. The compound to be tested was then added and fluorescence levels were recorded every 5 seconds for 60 seconds. The top and bottom rows of the plate were omitted from the assay to reduce edge effects on the results, and the first and last columns were used for controls.
The resulting fluorescence data was analyzed by dividing post-treatment fluorescence (each time point) by pre-treatment fluorescence from the same well, subtracting 1 and multiplying by 100 to give % increase in fluorescence.

**4.2.4 Cross-linking experiments**

**4.2.4.1 Cross-linking experiments between DmOrco and DmOr22a expressed in Sf9 cells**

Two flasks containing 150 mL at 2 x 10^6 cells/mL were infected either with His\textsubscript{10}-Flag-DmOrco alone at an MOI-1, or with both His\textsubscript{10}-Myc-DmOrco and Flag-DmOr22a at an MOI-1 each. The cells were incubated at 27°C for 72 hours with shaking at 100 rpm. The cells were pelleted at 3600 g and the pellet was resuspended in 25 mL of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl. Five aliquots of 5 mL each were incubated with the respective cross-linking reagent at a final concentration of 1 mM or 1% for PFA. The samples were rotated at room temperature for 45 minutes and the reaction was quenched with 50 mM Tris pH 7.5 for 15 minutes at room temperature. The His\textsubscript{10}-Flag-DmOrco samples were pelleted and lysed in buffer with 1% Zwittergent 3-16, mixed for 1 hour, then centrifuged at 100,000 g for 1 hour. The supernatant was removed and evidence of the formation of oligomers was assessed with coomassie stained SDS-PAGE gels. The cells expressing both His\textsubscript{10}-Myc-DmOrco and Flag-DmOr22a were pelleted and lysed in buffer with 1% Zwittergent 3-16, mixed for 1 hour, then centrifuged at 100,000 g for 1 hour. The supernatant was removed and subjected to Ni-NTA purification as in 3.2.4.3.

**4.2.4.2 Cross-linking experiments between DmOrco and DmOr67d expressed in HEK 293 cells**

HEK 293 T-REx-Myc-DmOrco/His\textsubscript{10}-V5-Or67d cells were cultured in 6 x T75 cell culture flasks to ~80% confluency and induced with 1 mM doxycycline overnight. The cells were pelleted at 3600 g and the pellet was resuspended in 5 mL of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl. Five aliquots of 1 mL each were incubated with the respective cross-linking reagent at a final concentration of 1 mM or 1% for formaldehyde. The samples were rotated at room temperature for 45 minutes and the reaction was quenched with 50 mM Tris pH 7.5 for 15
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minutes at room temperature. The cells were pelleted and lysed in buffer A with 1% Zwittergent 3-16, mixed for 1 hour, then centrifuged at 100,000g for 1 hour. The supernatant was mixed with 200 uL of Ni-NTA slurry (Qiagen) that had been pre-washed with buffer A containing 1% w/v detergent (Zwittergent 3-16). The sample was rotated for 1 hour then packed into a column, the flow-through was collected and the column was washed in ten column volumes of buffer B with 300 mM NaCl and 50 mM imidazole, and a further ten column volumes of buffer B with 150 mM NaCl and 50 mM imidazole. Protein was eluted with four column volumes of buffer B with 150 mM NaCl and 250 mM imidazole. An interaction between the subunits was evaluated by western blot against the epitope tag on each protein as described in section 2.2.5.

4.2.5 Single Molecule Fluorescence Photobleaching

4.2.5.1 Cloning for expression in T-Rex HEK 293 cells

Four odorant receptors from *D. melanogaster* were used in this study, DmOrco, DmOr22a, DmOr35a and DmOr43b. Each of the OR genes were tagged at the N-terminus with either eGFP or mCherry using a combination of PCR amplification and restriction enzyme digestion plus ligation. The eGFP-DmOrco construct was donated by Dieter Wicher (Wicher et al. 2008). This construct was modified for gateway cloning as described in German et al. (2013), and subsequently gateway cloned into the pT-Rex™-DEST30 vector (Life Technologies) using LR clonase II (Life Technologies).

PCR primers were designed to add a SacII restriction enzyme site to both the N- and C-terminus of eGFP and mCherry (SacII-mCherry/GFP-For and mCherry/GFP-SacII Rev), and a SacII restriction enzyme site to the N-terminus and an Apal restriction enzyme site to the C-terminus of the ORs (SacII-OrX For and OrX-Apal Rev) using the primers listed in Table 4-1. The primers were designed to remove the methionine-encoding ATG from the start of the ORs to prevent translation of untagged protein and insert a new methionine codon 5’ of the fluorescent tags. The eGFP and mCherry PCR products were inserted into pCR8/GW/TOPO vector (Life Technologies), digested with SacII restriction enzyme and gel purified using the QIAquick Gel Extraction kit (Qiagen). The OR PCR products were inserted into pCR8/GW/TOPO vector (Life Technologies), linearised with SacII restriction enzyme, gel
purified as before and dephosphorylated with Antarctic phosphatase (New England Biolabs). The purified eGFP and mCherry genes were ligated into the linearised OR constructs with T4 ligase (Life Technologies).

This resulted in the constructs with either SacII-eGFP-OR-Apal, or SacII-mCherry-OR-Apal. A NotI restriction enzyme site was then added to the N-terminus of these constructs via PCR amplification using the primer NotI-mCherry/GFP For. These PCR products were inserted into the pCR8/GW/TOPO vector (Life Technologies) digested with NotI and Apal restriction enzymes, gel purified and ligated into either the pcDNA4-TO or pcDNA5-TO (Life Technologies) vectors for expression in the T-Rex HEK 293 cell line. Plasmids were Sanger sequenced at Macrogen (Seoul, Korea) using plasmid-specific primers. DNA sequence analysis was performed using GENEIOUS Pro version 6.0.4 (Biomatters Ltd, New Zealand http://www.geneious.com).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SacII-Or22a For</td>
<td>5’-AATGGCCGCGGGTTAAGCAAGTTTTTTCACATTC-3’</td>
</tr>
<tr>
<td>SacII-Or35a For</td>
<td>5’-AATGGCCGCGGGTTAAGCAAGCTTTTGCGGCCTGATG-3’</td>
</tr>
<tr>
<td>SacII-Or43b For</td>
<td>5’-AATGGCCGCGGGTTAAGCAAGCTTTTGCGGCCTGATG-3’</td>
</tr>
<tr>
<td>SacII-Orco For</td>
<td>5’-AATGGCCGCGGGTTAAGCAAGCTTTTGCGGCCTGATG-3’</td>
</tr>
<tr>
<td>Or22a-Apal-Rev</td>
<td>5’-GCAATGGGCGCCCTATTGAGACCTTTGGCGG-3’</td>
</tr>
<tr>
<td>Or35a-Apal-Rev</td>
<td>5’-GCAATGGGCGCCCTATTGAGACCTTTGGCGG-3’</td>
</tr>
<tr>
<td>Or43b-Apal-Rev</td>
<td>5’-GCAATGGGCGCCCTATTGAGACCTTTGGCGG-3’</td>
</tr>
<tr>
<td>Orco-Apal-Rev</td>
<td>5’-GCAATGGGCGCCCTATTGAGACCTTTGGCGG-3’</td>
</tr>
<tr>
<td>SacII-mCherry/GFP For</td>
<td>5’-AATGGCCGCGGGTTAAGCAAGCTTTTGCGGCCTGATG-3’</td>
</tr>
<tr>
<td>mCherry/GFP-SacII Rev</td>
<td>5’-CGGTTCCGCGGCTTTGCTAGCTCGTCCTCATG-3’</td>
</tr>
<tr>
<td>NotI-mCherry/GFP For</td>
<td>5’-AATGGGCGCCGCGCATGAGACAGGCCG-3’</td>
</tr>
</tbody>
</table>

### 4.2.5.2 Expression of fluorescently-tagged OR subunits in HEK 293 cells

The HEK-293 cell line was stably transfected with pcDNA6/TR which constitutively expresses the tetracycline repressor protein (TR; Corcoran et al. 2013). Cell lines expressing the TR have the ability to regulate expression of genes present in other, Tetracycline-Operon (TO) containing plasmids such as pcDNA4/TO, pcDNA5TO and pTREx-DEST30 (Life Technologies).
2 x 10^6 TREX-HEK-293 cells were plated into a T25 flask and incubated overnight at 37°C with 5% CO₂. The cells were transfected with linearised DNA using Lipofectamine 2000 (Life Technologies) following the manufacturer’s instructions. The cells were incubated overnight and the media was removed, the cells were washed once with PBS and fresh media was added, and the cells were incubated for 6-8 hours at 37°C with 5% CO₂ before selective antibiotics (G418 500 µg/mL) were added. The media and antibiotics were refreshed every 2-3 days until the cells reached confluency.

Stable cell lines expressing either eGFP-DmOrco alone, eGFP-DmOrco/mCherry-DmOr35a or eGFP-DmOrco/mCherry-DmOr22a were prepared as described in section 4.2.1.2.1. To optimize the expression of functional tagged receptors these cell lines were single cell sorted on a Becton-Dickinson Aria II SORP Fluorescence Activated Cell Sorter (FACS), exciting the eGFP with a 488nm laser and mCherry with a 552nm laser. From this single cell sorting four isogenic cell lines were developed for eGFP-DmOrco and two for both eGFP-DmOrco/mCherry-DmOr35a and eGFP-DmOrco/mCherry-DmOr22a. These were tested for function using the Ca^{++} influx assay outlined in 4.2.3.

The TREX-HEK-293/eGFP-DmOrco, or TREX-HEK-293 cell line was plated into T25 flasks at 2 x 10^6 cells. These were incubated overnight and then transiently transfected with 8 µg of pcDNA4-TO vector containing the tagged constructs using Lipofectamine 2000 (Life Technologies) following the manufacturer’s instructions. The cells were incubated overnight at 37°C with 5% CO₂, the media was removed and the cells were washed once with PBS and lifted with TrypLE™ Select. Three hundred thousand cells were plated into each well of a 12-well plate containing a 12 mm round coverslip that had previously been incubated at room temperature with 50 µg/mL poly-d-lysine for three hours and washed twice with PBS. The cells were incubated for 6-8 hours at 37°C with 5% CO₂ then induced with 1 µg/mL doxycycline, and incubated overnight at 37°C with 5% CO₂. Each well was washed twice with PBS, and incubated at 4°C with hypo-osmotic buffer (4 mM PIPES, 30 mM KCl, pH 6.2) for 20 minutes (Hallworth and Nichols 2012). Cells on the cover-slips were lysed with a steady stream of hypo-osmotic buffer using a 25 gauge hypodermic needle attached to a 1 mL syringe. This resulted in predominantly outer membranes attached to the surface of the
coverslip, with very few whole cells remaining. Each coverslip was washed twice with hypo-osmotic buffer, mounted on a glass microscope slide and sealed with clear nail polish.

4.2.5.3 Fluorescence microscopy of insect ORs in HEK-293 cell membranes

Membrane fragments containing fluorescence were observed on a Nikon Eclipse TE2000-E inverted fluorescence microscope, via a 60X 1.20 numerical aperture objective. GFP excitation was performed using a halogen lamp with excitation at 482 nm and emission at 536 nm, and mCherry excitation was performed at 562 nm with emission at 624 nm. Fluorescent images were acquired using a Photometrics Evolve cooled back-illuminated electron-multiplying charged-coupled device (EMCCD) camera. Images were acquired typically for 1800 frames at 0.2 s/frame over a 128 x 128 pixel image field.

Images were imported into ImageJ as a sequence, and the first ten images were averaged to reduce noise levels. Regions of interest (ROI’s) containing putative single molecules were then selected with a 5x5 pixel square. The mean gray value for each ROI was measured across the sequence and exported to Excel. Background subtraction was performed by subtracting the average mean gray value of three ROIs placed away from the putative single molecules. The resulting data was filtered using a Chung-Kennedy algorithm implemented in R (Chung and Kennedy 1991). Due to the amount of noise in the data it was necessary to filter it to reveal discrete steps. The Chung-Kennedy filter provides an edge-preserving running average that enhances any steps in the data (Reyes-Lamothe et al. 2010). Briefly it calculates the mean and SD from two consecutive data sets of a given size, and reports the mean of the set that has the lower SD. If one of the sets includes a step it will have the lower SD and so its mean will be reported (Coffman and Wu 2012).
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4.3 Results

4.3.1 Identification of appropriate resin for pull-down assays

Two different immobilized metal ion affinity chromatography (IMAC) resins were tested for their ability to bind His\textsubscript{10}-tagged insect ORs. One hundred and eighty mL of Sf9 cells at 2\times10^6 cells/mL were infected with His\textsubscript{10}-Flag-Or22a virus at an MOI of 1. After solubilisation the supernatant was split in half and subjected to purification on a 1 mL column containing either; Ni-NTA (which uses Ni\textsuperscript{2+}) or TALON resin (which uses Co\textsuperscript{2+}), as described in section 4.2.2.1. Equal volumes of each flow-through, wash and elution fraction from the two resins were then analysed by western blot with an anti-Flag antibody as described in section 2.2.5 (Figure 4-2). The Ni-NTA resin bound more of the expressed protein and was used for all further experiments (Figure 4-2 A).

![Figure 4-2: Western blot of IMAC purified His\textsubscript{10}-Flag-DmOr22a using two different IMAC resins. Ni-NTA (A), or Talon resin (B). Lane numbers 1-9 indicate; 1 – flow-through, 2 – first wash step, 3 – second wash step, 4 – 9 – elution fractions.](image)

4.3.2 Pull down assays on OR subunits expressed using baculovirus in Sf9 cells

In order to detect homomeric and heteromeric interactions between insect OR subunits, uniquely tagged subunits were expressed in Sf9 cells either separately or together. The
His$_{10}$-tagged OR was then immobilised on Ni-NTA and evidence for an interaction was analysed by western blot.

When the subunits were expressed separately in Sf9 cells, the Flag-DmOrco subunit was only observed in the flow-through and wash lanes (Figure 4-3 lanes 4 and 5) and not in the elution fractions where it would be expected if an interaction were occurring. All His$_{10}$-tagged OR subunits bound to the Ni-NTA resin with high efficiency, an example is given in Figure 4-3 (lanes 7-9) where the His$_{10}$-Flag-DmOr22a subunit is only evident in the elution fractions and not in the flow-through or wash fractions.

While there is good expression of both proteins when co-expressed, the majority of the non-His$_{10}$-tagged protein is evident in the flow-through and wash fractions indicating that it is not binding to the His$_{10}$-tagged subunit when co-expressed (Figure 4-3 lanes 4 and 5, and Figure 4-4 right hand westerns). If an interaction were to occur both subunits should be evident in the elution fractions where only the His$_{10}$-tagged subunit is observed. I systematically tested placing the His$_{10}$-tag on the N-terminus of the OrX subunits with no evidence of an interaction (examples shown in Figure 4-4 A - His$_{10}$-Flag-DmOr22a with Myc-DmOrco, and B - His$_{10}$-Myc-DmOr10a and Flag-DmOrco), or on the N-terminus of the DmOrco subunit (Figure 4-4 C). I also investigated placing the His$_{10}$-tag on the C-terminus of the DmOrco and DmOrX subunits with no evidence of an interaction (data not shown).

![Figure 4-3: Western blot of pull down assays carried out on separately expressed His$_{10}$-Flag-DmOr22a and Flag-DmOrco subunits in Sf9 cells. Lane numbering denotes; 1 – His$_{10}$-Flag-DmOr22a flow-through, 2 – first wash step, 3 – second wash step, 4 – Flag-DmOrco flow-through, 5 – wash step, 6 – wash step, 7 – 11 – elution fractions.](image-url)
I then systematically investigated whether reducing the amount of salt in the buffer impacted the ability to demonstrate an interaction by carrying out the pull down assay in the presence of 100 mM, 50 mM or 10 mM NaCl. However, there was no evidence of an interaction under any of these conditions (data not shown). I have demonstrated in chapter 2 that the DmOrco subunit is soluble in a number of detergents that the OrX subunits are not soluble in. I tested whether there is the potential for DmOrco to pull down OrX subunits using DDM and Brij 35; however there was no evidence for an interaction and western blots demonstrated that the OrX subunit was only present in the insoluble pellet (data not shown).
Figure 4-4: Western blot analysis of pull down assays carried out on the subunits co-expressed in Sf9 cells. Westerns on the left are the His\textsubscript{10}-tagged subunit, on the right are the non-His\textsubscript{10}-tagged subunit. A) His\textsubscript{10}-Flag-DmOr22a and Myc-DmOrco, B) His\textsubscript{10}-Myc-DmOr10a and Flag-DmOrco, C) His\textsubscript{10}-Myc-DmOrco and Flag-DmOr35a. Lane numbering denotes; 1 – flow-through, 2 – first wash step, 3 – second wash step, 4 – 9 – elution fractions.

4.3.3 Pull down assays on wheat germ cell-free expressed subunits

4.3.3.1 Non-specific binding of wheat germ cell-free expressed protein

The receptors expressed in the wheat germ cell-free system appeared to be soluble as no precipitant formed after the translation reaction. The first pull-down experiments therefore, were carried out using the wheat germ expressed material directly from the reaction
without the addition of detergent. It was found that the dithiothreitol (DTT) in the reaction mix reduced the Ni$^+$ on the NTA resin, so to reduce the DTT concentration the reactions were diluted two fold in 50 mM NaH$_2$PO$_4$ 150 mM NaCl, pH 7.4 prior to the addition of the Ni-NTA slurry. These initial experiments showed a potential interaction between DmOrco and DmOr22a as both subunits were evident in the elution fractions from the Ni-NTA column (Figure 4-5 A and B (lanes 4-9)).

However, subsequent experiments where the non-His$_{10}$ tagged protein was expressed and purified alone demonstrated that the protein was non-selectively binding to the Ni-NTA resin (Figure 4-5 C and D). Solubilising the sample in Zwittergent 3-16 removed this non-selective binding (Figure 4-5 E and F). This would indicate that there is a component of the wheat germ cell-free reaction that promotes non-specific binding to the resin. This phenomenon was also observed with non-specific binding of the wheat germ cell-free reaction mix to the Ni-NTA chip using SPR (Figure 3-13).
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To remove any endogenous factors that might be affecting the binding of the protein to the Ni-NTA or to other proteins I decided to pellet the protein at high speed (> 100,000g) and resuspend it in a detergent based buffer for the pull down assays (Figure 4-6 lanes 2 and 5). I demonstrated that the receptors could be pelleted at high speeds removing the protein from the soluble fraction (Figure 4-6 lanes 3 and 6).
4.3.3.2 Pull down assays with wheat germ cell-free expressed OR subunits

I demonstrated that it was possible to express both subunits in the same cell-free reaction, and that both subunits were pelleted and resuspended after a high speed spin (Figure 4-7 A lane 1). However, there was no indication of an interaction on western blots after Ni-NTA purification, as only the His\textsubscript{10}-Flag-DmOr22a subunit can be observed in the elution fractions (Figure 4-7 A lanes 6-9).

When the subunits were expressed in separate reactions there was no interaction observed as only the His\textsubscript{10}-Flag-DmOr22a subunit is present in the elution fractions of the Ni-NTA purification (Figure 4-7 B lanes 9-14). The Flag-DmOrco subunit is observed in the flow-through and wash fractions (Figure 4-7 b lanes 6-7).
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Figure 4-7: Western blot of pull down assays carried out on the wheat germ cell-free expressed subunits. (A) co-expressed His$_{10}$-Flag-DmOr22a and Flag-DmOrco, lane numbering denotes 1 – reaction, 2 – flow-through, 3 – first wash step, 4 – second wash step, 5 – 9 – elution fractions. (B) separately expressed His$_{10}$-Flag-DmOr22a and Flag-DmOrco, lane numbering denotes 1 – His$_{10}$-Flag-DmOr22a reaction, 2 – Flag-DmOrco reaction, 3 – His$_{10}$-Flag-DmOr22a flow-through, 4 – first wash step, 5 – second wash step, 6 – Flag-DmOrco flow-through, 7 – wash step, 8 – wash step, 9 – 14 – elution fractions.

Identical results were obtained for each of the following combinations; DmOr10a/DmOrco, DmOr35a/DmOrco, DmOr43b/DmOrco, DmOr67d/DmOrco, DmOr10a/DmOr10a, DmOr22a/DmOr22a, DmOr43b/DmOr43b, DmOr67d/DmOr67d, DmOrco/DmOrco, and DmOr10a/DmOr22a (data not shown). The DmOr35a subunit however, could not be expressed with a His$_{10}$-tag and was only tested against the His$_{10}$-DmOrco subunit.

4.3.4 Pull down assays on HEK 293 expressed subunits

4.3.4.1 Functional assays

OR subunits expressed in the HEK 293 cell system were first assessed for function using a Fluo IV-based calcium influx assay (Corcoran et al. 2013). This assay demonstrated that Myc-DmOrco responds to VUAA1 (EC$_{50}$=11.1 µM), indicating that the DmOrco subunit was expressed in the plasma membrane and formed a functional ion channel (Figure 4-8).

The Myc-DmOrco/His$_{10}$-Flag-Or10a and Myc-DmOrco/His$_{10}$-Flag-Or67d cell lines were also tested for functionality using their ligands, methyl salicylate and cis-vaccenyl-acetate respectively. The Myc-DmOrco/His$_{10}$-Flag-Or10a cell line responded to methyl salicylate with an EC$_{50}$ of 44.6 nM (Figure 4-9). However, no activity was detected for the Myc-DmOrco/His$_{10}$-Flag-Or67d cell line when tested against cis-vaccenyl-acetate at concentrations from 5 nM to 100 µM in three different experiments (data not shown). To check if the cell lines were expressing the tagged protein, western blots were carried out
using an anti-Flag antibody for the DmOrX and an anti-myc antibody for the DmOrco subunit. Bands of the expected size (~50 kDa) were evident for the DmOrco subunit in the induced cells only (Figure 4-10). However, there were no bands evident in the blot for either the DmOr10a or the DmOr67d subunits (data not shown), even though the DmOr10a subunit was functionally active in this cell line. In this case the problem was thought to be due to cleavage of the Flag epitope by endogenous kinase activity, however this was not tested. Three subunits (DmOr10a, DmOr22a and DmOr67d) were re-cloned with a His\textsubscript{10}-V5 tag at the N terminus for expression in the HEK 293 cell line. The His\textsubscript{10}-V5-DmOr10a cell line responded to methyl salicylate at a similar level as the Myc-DmOrco/His\textsubscript{10}-Flag-DmOr10a cell line. However, the His\textsubscript{10}-V5-DmOr22a and His\textsubscript{10}-V5-DmOr67d cell lines did not respond to either methyl hexanoate or cis-vaccenyl acetate, respectively (data not shown).

![Figure 4-8](image)

Figure 4-8: Dose response curve from a calcium influx assay on Flp-In 293 T-REx cells expressing n-Myc-DmOrco. Cells were tested against a range of concentrations of VUAA1 from 50 µM to 10 nM. Fluorescence data was collected 30 seconds after the addition of ligand. Three wells of cells were tested for each concentration of agonist on induced (blue line) and uninduced (black line) cells. Vertical dashed line indicates the EC\textsubscript{50} value. Error bars represent standard errors of the mean of three replicates.
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Figure 4-9: Dose response curve from a calcium influx assay on Flp-In 293 T-REx cells expressing n-Myc-DmOrco/His$_{10}$-Flag-OR10a. Cells were tested against a range of concentrations of methyl salicylate from 4 µM to 2 pM. Fluorescence data was collected 30 seconds after the addition of ligand. Three wells of cells were tested for each concentration of agonist on induced (blue line) and uninduced (black line) cells. Vertical dashed line indicates the EC$_{50}$ value. Error bars represent standard errors of the mean of three replicates. The His$_{10}$-V5-Or10a construct responded to methyl salicylate at a similar level (data not shown).

Figure 4-10: Western blot analysis of Myc-DmOrco expressed in HEK 293 cells. HEK-293 cells expressing induced (lane 1) or uninduced (lane 2) Myc-DmOrco/His$_{10}$-Flag-DmOr10a, and induced (lane 3) or uninduced (lane 4) Myc-DmOrco/His$_{10}$-Flag-DmOr22a, were observed by western blot with an anti-Myc antibody.

4.3.4.2 Pull down assays on OR subunits expressed in HEK-293 cells

The non-His$_{10}$-tagged subunit was not evident in the elution fractions for any of the three construct pairs tested (Myc-DmOrco/His$_{10}$-V5-DmOr10a, Myc-DmOrco/His$_{10}$-V5-DmOr22a and Myc-DmOrco/His$_{10}$-V5-DmOr67d) when co-expressed or expressed separately (an example of the His$_{10}$-V5-DmOr67d and Myc-DmOrco pull-down is shown in Figure 4-11).
expression levels of the OR subunits were abundant enough to visualise on a western blot (Figure 4-11), however the V5 antibody produced a high background and the bands were often difficult to observe.

![Western blot of pull down assays carried out on co-expressed subunits in HEK-293 cells. Hi5-V5-DmOr67d and Myc-DmOrco were co-expressed in HEK-293 cells. A) anti-myc antibody, B) anti-V5 antibody. Lane numbering denotes; 1 – flow-through, 2 – first wash step, 3 – second wash step, 4 – 10 – elution fractions. The Myc-DmOrco subunit is only evident in the flow-through and wash step (lane 1 and 2) and not in the elution fractions where it would be expected if an interaction was occurring.]

4.3.5 Cross-linking Experiments

4.3.5.1 Cross-linking OR subunits in Sf9 and HEK-293 cells

To test whether the addition of cross-linking reagents could stabilise the potential complex formation of DmOrco and the DmOrX subunits in heterologous cells, His\textsubscript{10}-Myc-DmOrco and Flag-DmOr10a were expressed in HEK 293 and Sf9 cells. However, adding cross-linkers to heterologous cells prior to solubilisation of the OR subunits did not result in any non-His\textsubscript{10}-tagged subunit being observed in the elution fractions of the pull-down assays (Figure 4-12). The His\textsubscript{10}-Myc-DmOrco subunit is evident in lanes 4 and 5 for each treatment in Figure 4-12, indicating that it is binding to the Ni-NTA, however the Flag-DmOr10a subunit is only evident in lanes 1 and 2 for each treatment indicating that it is not interacting with DmOrco (Figure 4-12). Identical results were observed when the experiment was repeated with DmOr22a and DmOrco expressed in both heterologous cell systems (data not shown).
Figure 4-12: Western blot analysis of cross-linking experiments on insect OR subunits expressed in Sf9 cells. Sf9 cells were infected with both His<sub>10</sub>-Myc-DmOrco and Flag-DmOr10a virus. The infected cells were incubated with five cross-linking reagents and then subjected to Ni-NTA purification. Equal volumes of each sample were loaded on an SDS-PAGE gel. Lane numbers indicate 1) Flow-through, 2) first wash step, 3) second wash step, 4-5) elution fractions.
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4.3.6 Single Molecule Fluorescent Photobleaching

German et al. (2013) demonstrated that fluorescently-tagged insect OR subunits formed both homomeric and heteromeric complexes when expressed in heterologous cells. To determine the oligomeric state of these complexes single molecule fluorescent photobleaching experiments were carried out on the insect OR subunits expressed in HEK 293 cell membranes.

4.3.6.1 Full length expression of tagged OR subunits

The HEK-293-eGFP-DmOrco isogenic cell line was fluorescent upon induction with doxycycline when observed under a light microscope with the requisite filters. A band of the expected size for the full length fusion protein and the monomeric GFP were observed on a western blot using an anti-GFP antibody (Figure 4-13 lane 1). This result suggested the full length protein was being expressed, although a small amount of monomeric GFP was also evident. However, the isogenic cell lines developed for mCherry-DmOr22a and mCherry-DmOr35a (data not shown) did not express full length protein, there was some evidence for a monomeric mCherry in the western blot (Figure 4-13 lanes 1 and 2). This was likely due to the cell sorting process selecting cells that were expressing high levels of a truncated protein corresponding to the monomeric mCherry.

Figure 4-13: Western blot analysis of HEK 293 isogenic cell lines expressing OR subunits tagged with fluorescent proteins. 1) eGFP-DmOrco, 2) mCherry-DmOr22a.
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To determine if full-length DmOr22a, DmOr35 and DmOr43b could be expressed in HEK 293 cells, these constructs were transiently transfected and analysed by western blot. The transient mCherry-DmOrX, mCherry-DmOrco and eGFP-DmOrX constructs expressed well upon induction, and western blots with either anti-GFP or anti-mCherry antibodies demonstrated that full length fusion proteins were being expressed (Figure 4-14). The OrX subunits ran as a doublet on the western blots, this could be due to a number of factors including: glycosylation, protease activity or truncation.

![Western blot image](image)

*Figure 4-14: Western blot of HEK-293 cells transiently expressing fluorescent protein-tagged OR subunits. A) anti-mCherry antibody, B) anti-GFP antibody. Lane numbers indicate 1) mCherry-DmOrco/eGFP-DmOr22a, 2) mCherry-DmOrco/eGFP-DmOr35a, 3) mCherry-DmOrco/eGFP-DmOr43b.*

**4.3.6.2 Functional assays on fluorescently-tagged insect ORs**

To determine if the fluorescently-tagged OR subunits were forming functional complexes, they were tested for response to their respective ligands using the functional assay outlined in section 4.2.3. The eGFP-DmOrco cell line after single cell sorting responded to the agonist VUAA1 ($EC_{50}=7.6 \, \mu M$) (Figure 4-15), which is consistent with the Myc-DmOrco subunit, indicating the subunit was correctly folded and forming an ion channel in the HEK 293 cell membrane. This isogenic cell line was used for further single molecule photobleaching experiments.
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Figure 4.15: Functional assay on TREX-HEK 293 cells expressing eGFP-DmOrco. The isogenic cell line after single cell sorting was tested against VUAA1 at concentrations from 5 pM to 50 µM. The blue line represents induced cells, and the black line indicates uninduced cells. Vertical dashed line indicates the EC<sub>50</sub> value of 7.6 µM.

The cell lines transiently expressing the different OrX subunits were also tested for function using the calcium flux assay, but no increase in fluorescence above background levels was observed for a range of concentrations of their respective ligands (data not shown). This might be due to the presence of the large fluorescent tag on their N-termini interfering with either ligand binding or preventing activation of the DmOrco ion channel.

4.3.6.3 Microscopy of fluorescently-tagged insect ORs

The individual cell lines expressing the different fluorescently-tagged subunits were lysed using hypo-osmotic buffer and a 25 gauge needle, resulting in patches of lysed membranes that could be visualized using fluorescence microscopy. Uninduced TREX-HEK-293/eGFP-DmOrco cells were used as a negative control to ensure any fluorescence observed was due to the presence of tagged OR subunits. To reduce the possibility of pre-bleaching while searching for membrane patches and focusing on fluorescent regions, a neutral density filter was used. However this made it difficult to focus accurately on fluorescent regions, and resulted in poor focusing of some acquisitions. The images obtained were imported into ImageJ and all of the putative single molecules were analysed, an example acquisition is shown in Figure 4.16.
Each of the eGFP-DmOrX/mCherry-DmOrco datasets consisted of 6 slides from which between 150 and 200 ROIs were selected representing potential single molecules. However, the mCherry signal did not result in obvious single molecules and so it could not be ascertained as to whether both subunits were present in these ROI’s, therefore these datasets could not be analysed for the oligomeric structure of the OR complex.

The eGFP-DmOrco dataset consisted of 24 slides, from which 1065 different ROIs were selected representing potential single molecules. There were a small proportion of traces that showed obvious steps after filtering with the Chung-Kennedy algorithm (Figure 4-17 A and B), however, none of these traces bleached to background levels, and most of these showed a number of discrete steps interspersed with slow but steady bleaching (Figure 4-17 A, B and C). The majority of traces either; rapidly decreased and then leveled off (Figure 4-17 D), decreased at a slow but steady rate over the duration of the experiment (Figure 4-17 C), or did not bleach at all (Figure 4-17 E). None of the traces followed discrete steps to a background level. Extending the bleaching time did not reduce fluorescence to background levels. The lamp intensity settings were adjusted to increase the amount of bleaching occurring, but again had no effect on the rate of bleaching.

Figure 4-16: Representative image of putative single molecules of eGFP-DmOrco. A) The first ten frames of the sequence were averaged to reduce background noise, B) putative single molecules were selected with a 5x5 pixel ROI. Excitation was performed at 482nM with emission at 536 nM. Mean grey value for each ROI was calculated in ImageJ and exported to Microsoft Excel for analysis.
The traces from each of the potential single molecules were binned into one of five groups (Figure 4-17). These groups were defined as follows; multiple steps that were difficult to accurately count (Figure 4-17 A 1.8%), one or two discrete steps (Figure 4-17 B 0.9%), slow but steady bleaching with some apparent small steps (Figure 4-17 C 21%), rapid bleaching (Figure 4-17 D 54%) or no bleaching (Figure 4-17 E 22%). Less than 3% of the traces obtained could be binned into one of the first two groups which made it impossible to ascertain an accurate estimate of the number of GFP proteins in each molecule.
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Figure 4-17: Representative traces from putative eGFP-DmOrco single molecules. Each of the traces were binned into one of 5 groups: A) multiple steps that were difficult to count, B) one or two obvious steps, C) slow and steady bleaching with potential steps, D) rapid bleaching, E) no bleaching. The grey line represents the raw data, and the red line represents the output from the Chung-Kennedy filter. Arrows indicated potential GFP bleaching steps.

To reduce the apparent rapid bleaching of the GFP fluorescence observed in over 50% of the traces (Figure 4-17 D), TREX-293/eGFP-DmOrco cells were plated onto new coverslips and mounted onto slides using Prolong Gold Antifade mounting solution (Life Technologies)
which is designed to reduce photobleaching. However, this did not have any effect on the rate of bleaching of the GFP fluorescence (data not shown).
4.4 Discussion

In Chapter 3 I demonstrated that the insect odorant receptor subunits are forming higher order structures in Native PAGE gels, and in solution were predominantly forming monomers and dimers when analysed by SEC-MALS. To ascertain whether the insect OR subunits were interacting to form an ion channel complex when expressed in cell membranes, pulldown assays, cross-linking experiments and single molecule fluorescence photobleaching experiments were performed. Three expression systems were tested to look at this question; wheat germ cell-free expression, baculovirus-mediated expression in Sf9 cells, and an inducible HEK-293 system.

While others have found evidence for an interaction between insect OR subunits both in vivo and in heterologous cells (Neuhaus et al. 2005; Benton et al. 2006; Pask et al. 2011; German et al. 2013). I could not provide any evidence for an interaction using pull-down assays with either co-expressed or separately expressed subunits in any of the expression systems. The lack of evidence for an interaction could be due to several factors inherent in both the solubilisation strategy and the assay itself. The successful solubilisation of the subunits was only possible in the zwitterionic detergent, Zwittergent 3-16. There is very little data in the literature concerning the solubilisation ability of this detergent and it is possible that it is disrupting any potential interactions between the subunits. The denaturing abilities of a detergent are generally attributable to the type of head group and the chain length. However, there is no definitive set of rules that allow a priori assessment of each individual detergent; the denaturing ability is best discerned empirically. The subunits demonstrated a degree of folding when analysed with circular dichroism as shown in (Figure 2.5), so the Zwitergent 3-16 is not completely denaturing the proteins. However, it could be disrupting the OR complex, or preventing it from forming in the case of the separate expression studies. Only a single publication using a co-immunoprecipitation approach has demonstrated an interaction between insect OR subunits (Tsitoura et al. 2010). In this case DDM was used as the solubilising detergent, which was unable to solubilise any of the OR subunits in this study. My study provides more evidence that an empirical approach must be taken to find the correct detergent and conditions to allow an interaction to take place.
The receptors expressed in either the wheat germ cell-free system or Sf9 cells lack a functional assay, which could confirm correct folding. It is possible that the protein being produced in these systems is not folded in a manner that allows interactions to occur. Analysis of folding showed that the protein produced in Sf9 cells has some secondary structure, but this does not imply that it is folded into its correct tertiary or quaternary structure. The use of baculovirus-mediated expression in Sf9 cells has been demonstrated to express membrane proteins predominantly in internal membranes (Vialard et al. 1990; Cserepes et al. 2004; German et al. 2013), where it might not be functional as it is has not been trafficked to the plasma membrane. The level of functional protein expressed in heterologous cell systems is dependent on many factors including, the amount and quality of mRNA synthesised, folding of the peptide chain in the ribosome and translocon, post-translational modification, and chaperones to ensure correct folding (Andrell and Tate 2013). The large amounts of protein produced in the Sf9 system could cause over-loading of cellular machinery resulting in incorrect folding or incorrect insertion of the receptor subunits in the cell membrane. It is important to note that cell membranes are not inert physical barriers, but complex environments with dynamic properties that affect membrane protein structure and function, and their ability to form stable interactions. The heterologous cells and cell-free system might not provide the necessary membrane properties to replicate the in vivo environment that induces the OR complex formation. An alternative expression system for this experiment to increase the probability of the OR subunits interacting, would be to create transgenic D. melanogaster flies expressing epitope-tagged ORs that could then be subjected to pull down assays. However, it would still be necessary to screen detergents and buffers to identify optimum conditions for protein solubilisation while maintaining the structure of the complex.

This study has demonstrated that at least two subunits of the insect OR complex are functional when expressed in HEK 293 cells. The DmOrco subunit was activated by VUAA1, and when expressed with DmOr10a the ion channel complex was activated with methyl salicylate. Although the D. melanogaster pheromone receptor (DmOr67d) can be expressed in all three systems it did not respond to the pheromone component cis-vaccenyl acetate in the calcium influx assay. This is likely due to the lack of the sensory neuron membrane
protein (SNMP1) which has been shown to be necessary for pheromone reception both in vivo and in heterologous cells (Benton et al. 2007; Jin et al. 2008). The SNMP1 protein might also be necessary for an interaction to occur between the DmOr67d and DmOrco subunits, perhaps forming a large receptor complex. It would be interesting to add this protein into the assay to investigate if the receptor is active in this cell line. The lack of observable Flag-tagged subunits on western blots when expressed in HEK 293 cells was thought to be due to endogenous kinase activity cleaving the Flag tag from the protein. To overcome this problem three subunits (DmOr10a, DmOr22a and DmOr67d) were re-cloned with a His$_{10}$-V5 tag at the N-terminus for expression in the HEK 293 cell line, each of these constructs could be visualised using an anti-V5 antibody on western blots, although only the DmOr10a construct responded to its ligand in the functional assay.

If the choice of detergent was the reason why the interaction could not be seen with the pull-down assay it would be expected that cross-linking the subunits prior to detergent solubilisation might solve this. However, this was not observed when tested with DmOrco and either DmOR10a or DmOr22a. This lack of cross-linking might be due to the choice of cross-linking reagents, even though they were chosen based on their ability to cross-link membrane proteins from the literature.

At the time that Chapter 2 was written and published, the TET inducible system was not available in my lab. I had attempted to express the subunits in HEK 293 cells using a transient non-inducible approach; however the expression levels were too low to allow further experiments. For this reason I have not yet carried out a full detergent screen on the subunits produced in the HEK 293 cell line. There may therefore be a detergent in my panel that solubilises both subunits from HEK 293 cells and maintains the complex in a way that can be visualised through Ni-NTA pull down assays.

The DmOrco and one DmOrX subunit was demonstrated to be functional in HEK 293 cells (Figure 4-8 and Figure 4-9), and thus it is likely that a heteromeric complex is forming in the membrane. Tagging membrane proteins with a GFP molecule allows the analysis of stoichiometry of complexes to be measured by the number of discrete steps taken to bleach the GFP molecule to background levels. The receptors were re-cloned with GFP molecules
on the N-terminus and subjected to photobleaching with fluorescent light and appropriate filters. However, the results did not match those achieved by other researchers who have reported very obvious and discrete steps with bleaching to background levels (Figure 4-18). The fluorescence bleaching was erratic and none of the putative single molecules bleached to background levels. Although there were discrete steps in some of the photo bleaching profiles, the exact number was difficult to calculate as the steps were interspersed with regions of either slow but steady bleaching or wildly fluctuating fluorescence levels.

This result is most likely due to the experimental setup and the specifications of the microscope lens that was used in the study. The lens used in this study was a 60 x 1.2 NA (numerical aperture) oil immersion lens, whereas those used in the literature are 100 x with a numerical aperture ranging from 1.4 to 1.65. The low magnification and NA value reduced the ability to detect single molecules in the immobilized membranes and also increased the level of background noise. The NA is effectively a measure of the objective’s ability to resolve fine detail, and thus the lower the NA value the lower the resolution of the images. The fact that the majority of traces were bleached rapidly might be due to the relatively high power of the lamp used even on its lowest setting. In an attempt to mitigate this rapid bleaching, the samples were mounted on slides using anti-fade mounting reagent, designed to reduce photobleaching. However this did not reduce the speed at which the bleaching occurred. The power output of the lamp may be too high for single molecule photobleaching experiments.

Most groups utilise TIRF microscopy to analyse the stoichiometry of membrane proteins as this allows a thin region of the specimen (usually within 200 nm of the cell membrane) to be observed. This removes the need to lyse the cells and mount membranes on coverslips, thus allowing the protein complex to remain in a native state. I did not have access to a TIRF microscope and instead followed the procedure of Hallworth et al. (2012), who demonstrated that Prestin molecules form tetramers in HEK cell membranes. There are other factors such as, optical components out of alignment and poor filter sets that can lead to a low detection threshold. The danger of such a detection threshold is that only the aggregates make it over the limit, and thus single molecules are never detected (Brouhard 2010). This might explain why I have a large number of traces with a large number of steps,
as the aggregated protein may still bleach in a step wise manner, but with aberrant or exaggerated properties.

Figure 4-18: Single molecule photobleaching trace from Ulbrich et al. (2007), showing two obvious steps required to bleach the GFP-tagged molecule to background levels.

The DmOrco subunit remained functional when a GFP tag was attached to the N terminus. This would indicate that the N terminus region might not be required for Orco to form a homomeric ion channel. The mCherry-tagged OrX subunits however, were not functional in a calcium based assay when co-expressed with the GFP-DmOrco. This could be due to the placement of the tags interfering with either the heteromeric ion channel formation or the ligand binding region.

The inability of my pull down assay to discern an interaction between the insect OR subunits, might be due to problems inherent in the assay itself as discussed. However, there is also the possibility that the receptors do not form an interaction in these systems that is strong or stable enough to be examined using a biochemical approach. The cross-linking experiments were designed to ‘fix’ any interaction and should have made it stable enough to be visualised by western blot, however this did not occur. This could be taken as evidence that there is no stable interaction between the subunits and the receptor complex is not a heteromeric ligand gated ion channel, but rather a stand-alone receptor complex that interacts with second messenger systems to open other ion channels. Although there is evidence from the literature that the OR subunits interact (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013), and that both subunits contribute to the ion conducting properties of the channel (Pask et al. 2011), there is no evidence to indicate that this is a
stable interaction or that the two subunits form a stable heteromeric complex \textit{in vivo}. Experiments carried out in the Hawkmoth \textit{Manduca sexta} suggest that there is no Orco/OrX heteromeric ion channel, rather Orco is an ion channel that regulates the kinetics of the odorant response and the detection threshold (Stengl 2010; Getahun et al. 2013; Nolte et al. 2013). If this is the case there may not be a stable interaction between the subunits, instead they could be coupled through second messenger systems. The OrX subunits are known to interact with G\(\alpha\) subunits when coexpressed in heterologous cells, which in turn activate downstream messenger systems resulting in the opening of endogenous ion channels. Orco has been demonstrated to form an ion channel in heterologous cell systems, and it enhances the odour-evoked responses of OrX subunits when expressed together. However, it is not known whether the increase in sensitivity of OrX when expressed with Orco is due to increased amounts of receptor inserted in the cell membrane or because a heteromeric ion channel is formed. There is a large body of work in the literature that demonstrates the ability of insect ORs to signal through ion channels when expressed in heterologous cell systems (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008; Jones et al. 2011; Pask et al. 2011; Chen and Luetje 2012; Jones et al. 2012). However, there is no direct evidence that the receptors themselves form a heteromeric ion channel. It is possible that the receptors are signalling through a secondary messenger pathway which is in turn activating ion channels such as Orco.

Although this study has not provided evidence for a stable interaction between the insect OR subunits it should not be taken as evidence that an interaction does not occur \textit{in vivo}. It is difficult to come to any conclusions based on a negative result and further work is needed to investigate the possibility of this interaction occurring. However, the techniques applied in this study represent valid approaches to investigate this interaction, perhaps modified to include other detergents and cross-linking reagents.
Screening Crystallisation Conditions for Insect Odorant Receptors
Chapter 5: Screening Crystallisation Conditions for Insect Odorant Receptors

5.1 Introduction

The generation of high resolution crystal structures are an important first step in the analysis of the structural properties of a protein or protein complex. They provide an initial assessment of the three dimensional organisation of the protein and its atomic structure. However, membrane proteins have inherent characteristics that make them difficult to crystallise. A large portion of their surface is hydrophobic which means they are generally maintained in a detergent or lipid-based buffer which can impair the crystallisation process. They are also often unstable in solution, and can have large flexible areas that are difficult to resolve.

It is estimated that 20-30% of genes in most genomes encode membrane proteins (Wallin and von Heijne 1998; Krogh et al. 2001). However, of the more than 92,000 crystal structures in the PDB database less than 1% are from membrane proteins. There are 409 unique protein structures in the ‘Membrane proteins of known 3D structure’ database (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Of these, 22 are potassium channels and 16 are other ion channels. Membrane proteins are severely under-represented in the crystal structure databases.

The structure of the first membrane protein was published in 1985 (Deisenhofer et al.), with the first mammalian GPCR in 2000 (Palczewski et al.). The rate at which membrane protein structures are being solved is increasing with the advent of new techniques, screens that are based on conditions that have led to membrane protein structures, and the increase in the number of labs working in this field (Figure 5-1).
New systems are being developed and improved to overcome the numerous barriers to successfully solving the crystal structure of membrane proteins. These barriers include; the choice of expression system, the detergent used to solubilise and stabilise the membrane protein, improving protein stability through mutant screens, deletions, and fusion partners, as well as advancing the technologies behind automation of screening protocols and synchrotron beam line developments (Moraes et al. 2013).

Protein crystallisation is a process of screening a large number of conditions to assess their ability to allow crystal formation for the protein of interest. When initial conditions have been found fine screens can be employed to explore related conditions that optimise the size or stability of the crystals and ultimately allow the generation of crystal structure at high resolution. Often the detergent used during the crystallisation process also has to be screened to find a suitable condition (Privé 2007). This detergent might not be the same as that used in the solubilisation process. The choice of detergent can be a critical factor in obtaining high resolution data as each detergent has a different micelle size which changes the amount of protein exposed within the crystal. Crystallisation of membrane proteins can also be achieved using lipid phases such as cubic phase, sponge phase or bicelles instead of detergents (Parker and Newstead 2012).

Figure 5-1: Chart of the number of unique membrane protein structures added to the database by year, and the cumulative total. Bars indicate unique structures added by year (left Y axis), the black line indicates the cumulative total (right Y axis). Data from http://blanco.biomol.uci.edu/mpstruc.

![Figure 5-1: Chart of the number of unique membrane protein structures added to the database by year, and the cumulative total. Bars indicate unique structures added by year (left Y axis), the black line indicates the cumulative total (right Y axis). Data from http://blanco.biomol.uci.edu/mpstruc.](image-url)
The first step in crystallising a membrane protein involves finding the buffer conditions that allow the protein to be purified to homogeneity in a folded form. I have already demonstrated in chapter 2 how this can be carried out for insect ORs. However, the detergent that is most useful for solubilising a membrane protein is not always the most efficient at allowing crystals to form, and a further detergent screen might need to be carried out to discover more conditions that enhance the probability of obtaining crystals.

Current knowledge about the structure of the insect ORs is based on in silico predictions from the primary amino acid sequence, epitope tagging experiments in vivo and in vitro, and YFP fusion experiments in vivo (Benton et al. 2006, Smart et al. 2008). These indicate that DmOrco has a large intracellular loop (ICL) 2 that connects TM 4 and 5, and is not present in the ligand-binding ORs. The C-terminal portion of the insect ORs has been found to have several important domains including a potential ion-selectivity filter in TM6 (Benton et al. 2006), and three highly conserved regions (Miller and Tu 2008). Nichols and Luetje (2010) demonstrated that the extracellular half of TM3 in DmOr85b was involved in odorant activation of the receptor, and a conserved aspartic acid residue in TM7 of DmOrco influences sensitivity to VUAA1 (Kumar et al. 2013). A bioinformatics approach has also been taken to model the oligomeric structure of DmOrco alone and in a heteromeric complex with Or22a (Harini and Sowdhamini 2012). The authors predicted that the DmOrco subunit could form a tetramer similar to that found in the potassium channels, and based on this structure there are two possible ion channel pathways; one formed by TM4-5 with an intracellular pore forming domain, and the other formed by TM5-6 with an extracellular pore forming domain. However, the authors state that a full length model of the tetrameric DmOrco was difficult to predict due to a lack of templates for the loop regions in the structure databases. This type of modelling study is dependent on the type and quality of related proteins in the structural database, and in the case of the insect ORs there are currently no crystal structures available.

A crystal structure of a representative of the insect OR family would have an enormous impact on the field. It would allow for hypotheses to be tested around the potential signalling mechanism and/or channel formation, gain an insight into the stoichiometry of the complex and allow for examination of the potential ligand binding sites.
5.1.1 Aims

The aim of this chapter is to initiate crystal trials of the purified insect odorant receptor subunits.

I have already outlined the expression and purification protocols for these receptors in Chapter 2, and demonstrated the recombinant DmOrco and DmOr22a are folding into predominantly alpha helical structures (section 2.3.4). SEC-MALS analyses indicate that they are predominantly monomeric with a small amount of potential dimers (Section 3.3.3).

In this chapter I aim to screen each of the OR subunits for crystal formation using five commercially available crystallisation screens. If these initial screening conditions are successful they will be expanded to further test the ‘crystallisation space’ around them. This will include modifying the pH, the concentration of detergent, salt, protein, and other additives present in the initial screen.
5.2 Methods

The recombinant OR subunits (DmOr10a, DmOr22a, DmOr43b, DmOr67d and DmOrco) were expressed and purified as outlined in section 3.2.1. Each of the subunits was concentrated to at least 2 mg/mL using a 100,000 MWCO Vivaspin concentrator. The N-terminal His\textsubscript{10} epitope tag was removed by incubating the protein overnight with 1 mM DTT, 10 mM EDTA and 1.6 µM Tobacco Etch Virus protease at 18°C.

To remove the phosphate from the solubilisation buffer, which is known to form crystals under screening conditions, concentrated protein was exchanged into 20 mM Tris, 150 mM NaCl, 0.2 mM Zwittergent 3-16 on a gel filtration column. The peak fractions were collected and concentrated again to at least 2 mg/mL.

The crystallisation screens (MemGold, MemGold2, Morpheus, Structure Screen I + II and JCSG) containing 96 conditions each were purchased from Molecular Dimensions, aliquoted into 96 well deep-well plates and stored at 4°C. For each screening condition 200 µL was placed into 1 well of a 96 well flat bottom plate with a Beckman 3000 liquid handling robot. Protein drops were placed on the underneath of an adhesive PCR sealing film at 1 µL per drop and mixed with 1 µL of the respective screen condition. The sealing film was then inverted and placed on top of the 96 well plate containing the screen conditions and sealed. The plate was stored at 18°C, and checked regularly for crystal formation.

Images of the crystals were taken with an INFINITY2-3 digital CCD camera (Luminera) attached to a Leica MZFLIII microscope.
5.3 Results

The subunits were expressed and purified as in section 3.2.1. The subunits are forming predominantly monomers in the buffer and detergent conditions that I am using, with some evidence for a small amount of dimer formation (section 3.3.3).

Each of the subunits could be concentrated to ~2 mg/mL, however the ligand binding subunits would occasionally precipitate upon concentrating. When the protein precipitated it was disposed of and the purification steps were repeated.

The His$_{10}$ and epitope tags were removed with TEV protease enzyme, an example of the DmOrco subunit is shown in Figure 5-2 A. The protein was then exchanged into a Tris-based buffer on a gel filtration column, see example for the DmOrco subunit in Figure 5-2 B.

![Figure 5-2: Cleavage of the His$_{10}$ tag with tobacco etch virus protease. A) Coomassie-stained gel of the purified DmOrco subunit pre- (lane 1) and post- (lane2) cleavage of the His$_{10}$ tag with TEV protease. B) SEC profile of DmOrco after cleavage of the His$_{10}$ tag and exchange into 20 mM Tris, 150 mM NaCl and 0.2 mM Zwittergent 3-16. Blue line indicates DmOrco profile, and dashed line indicates MW standards with peaks from left to right of, 444 kDa, 200 kDa, 66 kDa, 29 kDa and 13.7 kDa. The Y axes correspond to the UV A280 readout for the DmOrco subunit (left), and the standards (right).]

The amount of protein purified for each subunit varied and as each condition required protein at 2 mg/mL, not all subunits were screened with all 5 crystallisation screens.
Preference was given to the two membrane protein screens MemGold and MemGold 2. Then the other three screens were initiated if enough protein was available. Only the DmOrco subunit was tested with all 5 screens.

DmOrco was the only subunit to produce crystals at the time that this chapter was written. Of the 480 conditions initially trialled, only one resulted in crystal formation; 0.1 M sodium acetate pH 4.6, 2 M ammonium sulphate (Figure 5-3). These crystals formed within three months of initiating the screen. The crystals were small thin plates, approximately 40 microns across (Figure 5-3), and did not increase in size over the duration of this study. A further screen was designed around this condition to explore the possibility of crystals forming more readily in slight variations of the original condition, and potentially growing to a size that could be tested for diffraction using an X-ray source.

Figure 5-3: Image of the initial crystals formed in the presence of 2 mg/mL DmOrco. There was only 1 crystal that presented a morphology shown by arrow A, the rest of the crystals presented with a triangular pyramid morphology such as those shown by arrow B.

The initial condition was expanded by screening the pH of the sodium acetate from pH 4 to pH 5.32, the concentration of ammonium sulphate from 1.7 M to 2.2 M, and two protein
concentrations; 2.2 mg/mL and 1.1 mg/mL. This resulted in the formation of crystals in 7 different conditions (Figure 5-4).

Figure 5-4: Crystals of DmOrco formed in extended conditions. A) 0.1 M sodium acetate pH 4.52, 1.7 M ammonium sulphate, 1.1 mg/mL protein, B) 0.1 M sodium acetate pH 5.02, 1.9 M ammonium sulphate, 2.2 mg/mL, C) 0.1 M sodium acetate pH 5.02, 2 M ammonium sulphate, 2.2 mg/mL protein, D) 0.1 M sodium acetate pH 5.32, 2 M ammonium sulphate, 2.2 mg/mL, E) 0.1 M sodium acetate pH 5.32, 1.9 M ammonium sulphate, 1.1 mg/mL protein, F) 0.1 M sodium acetate pH 5.32, 1.9 M ammonium sulphate, 2.2 mg/mL, G) 0.1 M sodium acetate pH 5.32, 1.8 M ammonium sulphate, 1.1 mg/mL.
None of the crystals formed were large enough to test for diffraction on our in-house X-ray source. However, 2 of the crystals were sent for testing in a micro-focus beam line at the Australian Synchrotron. The results indicated that these crystals do not contain any protein and are more then likely composed of either salt or detergent.
5.4 Discussion

The ability to produce insect ORs in a monomeric form with evidence that they maintain some secondary structure allowed the initiation of crystal trials on this purified material. Although six ORs were originally used in this study, only five could be expressed with a His<sub>10</sub>-tag to levels sufficient for purification (DmOrco, DmOr10a, DmOR22a, DmOr43b and DmOr67d). Of these five ORs the DmOrco subunit proved to be the most amenable to structural studies for several reasons; it expresses at higher levels than the other subunits, it produces a more monodisperse SEC profile with little aggregation evident, it remains stable in solution at room temperature for extended periods and it was the only subunit to produce crystals at the time of writing.

The DmOrco subunit was initially screened using five commercially available screens, Morpheus, JCSG+, Structure Screen 1&2, MemGold and MemGold2. The first crystals were formed in 0.1 M sodium acetate pH 4, 2 M ammonium sulphate, 2 mg/mL DmOrco. This initial screen was expanded in both pH range and ammonium sulphate concentration and resulted in the formation of 7 more crystals.

The crystals that were formed in this study have not yet been placed in an X-ray beam and it is not known if they diffract. The crystals are too small for our in-house X-ray beam, and I do not have access to a microscope with UV filters to examine for potential tryptophan fluorescence. It is possible therefore, that these crystals are made up of either detergent or salt and do not contain any protein. The next step in this study will be to test for diffraction and expand the initial screening conditions to further examine the crystallisation space for these receptors.

One of the major stumbling blocks in this study was the production of stable protein that could be concentrated to a high level. The highest concentration achieved was 4.8 mg/mL for the DmOrco subunit. It was extremely difficult to concentrate the DmOrX subunits above 2 mg/mL. Although there are no definite rules for protein concentration in crystal trials, and it is generally accepted that the concentration required to produce crystals varies from protein to protein. It would be preferable to start at a relatively high concentration such as
10 mg/mL and dilute down if necessary. It might be possible to concentrate the insect ORs further by the addition of stabilising compounds such as glycerol.

This chapter outlines the initial screens for crystal production for five insect ORs. To further explore the possibility of generating a high resolution crystal structure from one of these receptors there are several different approaches that could be taken.

The initial step in a crystallisation experiment is obtaining a protein that is most likely to generate a high resolution crystal structure. This entails purifying the protein to homogeneity, ensuring it is able to be concentrated and remain stable. The protein can be tested in a variety of different buffers and detergents to determine the best conditions to meet these demands. Prior to initiating crystallisation screens various homologues or mutants of the protein can be tested for both stability and homogeneity.

It has been demonstrated that there is a correlation between the thermostability of membrane proteins and their ability to form crystals that diffract at high resolution (Sonoda et al. 2011). The thermostability of a protein refers to the temperature at which it unfolds. This can vary depending on the buffer conditions and the choice of detergent. The higher the temperature at which a protein unfolds the better the chances of obtaining a high resolution crystal structure. This was demonstrated with the turkey β₁-adrenergic receptor, which was modified by alanine scanning mutagenesis to provide a protein with a melting temperature (Tₘ) 21°C higher than that of the wild type (Serrano-Vega et al. 2008), and a crystal structure that was resolved to 2.7 Å (Warne et al. 2008). Sonada et al. (2011) went further to test if there was a level of stability above which a membrane protein has to be for resolution of a crystal structure. They used an unfolding assay which measured the length of time taken to unfold the protein at 40°C in several different detergents using the dye N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]-maleimide (CPM), which fluoresces upon binding the free sulfhydryl groups on cysteine residues. The cysteine residues in membrane proteins are predominantly found in TM regions, when the protein unfolds and the hydrophobic regions are exposed the fluorescence level increases (Alexandrov et al. 2008). They found that membrane proteins with an unfolding rate of longer than 17 minutes at 40°C can be considered stable enough to initiate crystal trials in that detergent. The
predicted cysteine residue positions for the insect Ors shows that they are relatively evenly distributed throughout the proteins. However, each of the five ORs have between 2 and 7 cysteine residues within the predicted transmembrane domains which might be enough to register a change in fluorescence using the CPM assay (Alexandrov et al. 2008; Sonoda et al. 2011). The CPM assay could be used as a rapid method to screen a range of detergents for their potential crystal formation abilities, and should be considered as a tool for any further experiments.
The homogeneity of the protein can be examined by monitoring the sharpness and symmetry of the SEC profile under various buffer conditions, and with a range of detergents (Kang et al. 2013). A method known as fluorescence size exclusion chromatography (FSEC) is a rapid tool to analyse the monodispersity of a protein sample as it does not require prior purification of the protein saving time and resources (Drew et al. 2001; Drew et al. 2005;
Drew et al. 2006). This method involves tagging the protein of interest with a GFP molecule, and measuring the GFP fluorescence as the protein passes through a gel filtration column. The symmetry of the resulting GFP fluorescence profile can then be used as a proxy for the monodispersity of the protein. Heating the samples at a range of temperatures prior to injection on the FSEC column allows analyses of the stability of the protein at the same time as the monodispersity (Hattori et al. 2012). These methods require much less protein than standard methods, often providing data for nanogram or microgram quantities of unpurified protein (Kawate and Gouaux 2006; Hattori et al. 2012). I have already generated GFP-tagged OR constructs for the single molecule photobleaching experiments in Chapter 4, and the use of FSEC for rapid analysis of different buffer and detergent conditions would be an excellent next step in the production of crystal structures for these proteins.

The two methods mentioned above allow for relatively rapid screening of proteins prior to initiating large scale purification and crystal trial experiments, and to choose those proteins that are most stable. The question then becomes how to increase the stability of the protein being studied? There are several methods employed to answer this question, from the use of high affinity ligands or inhibitors, small molecule addition, detergent screens, and mutagenesis (Kang et al. 2013). In the absence of a high affinity ligand, the use of mutagenesis or directed evolution has provided a valuable tool in the production of high resolution crystal structures of membrane proteins (Magnani et al. 2008; Serrano-Vega et al. 2008). However this approach is time and resource intensive and other approaches to increase the thermostability of the protein may be more applicable for smaller labs.

When the optimum conditions for protein stability and monodispersity have been found, initial crystallisation conditions can be screened. The crystals that are formed in these initial conditions can be tested for the presence of protein using a UV filter on a light microscope, as the tryptophan residues can be excited at 280nm. If the crystals are large enough they can also be tested for diffraction in an X-ray beam. These initial conditions should then be optimised.

The development of new detergents and new types of amphiphiles provides an important tool for protein crystallography. These new detergents may provide better conditions for
crystal formation, as well as providing a larger exposed surface for the production of high resolution crystal structures (Chae et al. 2010; Rasmussen et al. 2011; Rosenbaum et al. 2011). Additive screening is another important method to increase the probability of producing a high resolution crystal structure. Parker and Newstead (2012) found that all types of alpha helical membrane proteins benefited from the addition of some form of additive, with multivalent salts and polyalcohols appearing to be particularly advantageous (Moraes et al. 2013). The use of secondary detergents as an additive accounted for 19% of crystal structures reported in 2012 (Parker and Newstead 2012), making secondary detergent screens an important tool for optimisation experiments.

The stability and monodisperisty of a protein provides vital information about the potential crystal structure formation under particular conditions. However the production of crystals is often just a first step in the generation of high resolution structures. Often crystal optimisation has to take place to ensure diffraction at a high enough resolution to generate a useable structure. Crystal packing is mostly due to the interaction of hydrophilic regions within the proteins, the larger the exposed hydrophilic region the more likely it is to produce a structure at high resolution (Kang et al. 2013). One method to increase the size of the hydrophilic region is to screen detergents with a smaller micelle size in one of the methods described above. Another approach is the use of scaffold proteins. The addition of Fab or Fc fragments from monoclonal antibodies raised against the protein of interest is the most successful scaffold protein technique as it not only increases the surface area of the exposed regions it also acts to stabilise the protein (Kang et al. 2013). Scaffold proteins can also be fused to the protein of interest producing chimeric proteins, the T4 lysozyme has been used in this manner to generate high resolution crystals of several GPCRs (Cherezov et al. 2007; Jaakola et al. 2008; Warne et al. 2008). Although I have not attempted to develop antibodies against the insect ORs, it would be possible to do this; however, it might also be possible to use antibodies against the epitope tags that have been added to the N terminus of each receptor (Roosild et al. 2006; Pai et al. 2011).

In this chapter I have used a hanging-drop vapour diffusion method to initiate crystal formation. There are however, other methods that have been successfully used to resolve the structure of membrane proteins. Lipidic phase crystallisation techniques have been used
successfully to solve the structure 38 membrane proteins to date (http://cherezov.scripps.edu/structures.htm). Lipidic cubic phase provides a more native membrane-like environment for membrane proteins than detergent micelles. It also produces crystals with type I packing (Figure 5-6), this allows the protein molecules to make contacts through both the hydrophilic and hydrophobic portions resulting in better crystal ordering.

Type II crystals are commonly observed with detergent micelle based techniques such as those used in this study. In these crystals the lattice contacts are made primarily by the hydrophilic regions as the hydrophobic areas are surrounded by detergent molecules. The majority of membrane protein structures solved have been generated from type II crystals (Kang et al. 2013). However, regardless of the technique used to generate the protein crystals, high resolution X-ray diffraction data will depend on the quality of the protein-protein interactions, which can be improved with the stabilisation and scaffolding techniques described above.
Figure 5-6: Schematic representation of two types of membrane protein crystals. Type I are two-dimensional sheets of membrane-like crystals stacked in the third dimension. In type I crystals, lattice interactions are of both a hydrophilic and hydrophobic nature. This type of packing is found in the crystals grown from lipidic cubic phases. Type II crystals are formed by protein–detergent complexes and the lattice contacts consist primarily of interactions between the hydrophilic regions of the membrane protein. Modified from Michel (1983).

Generating a high resolution structure of an insect OR would provide a model for the prediction of both the receptor complex formation and ligand binding. It would also be of assistance in the search for insect repellents and pesticides which is extremely important for both human health and crop management. Within the scope of this chapter I have successfully screened initial conditions that have generated crystals for an insect odorant receptor. However, the formation of crystals is only an important first step in the generation of a high resolution structure. There is still a large amount of work to be done with these receptors before a structure will be forthcoming; although with the new screening tools and techniques that are available it is likely that a structure will be produced in the near future.
Final Discussion
6.1 Summary of Results

The structure and function of the insect odorant receptor complex is still not fully understood. However, recent advances point to the complex forming a ligand-gated non-selective cation channel. The current weight of evidence points to the complex comprising at least two subunits, the ubiquitous Orco and one OrX subunit which confers ligand-binding ability. However, nothing is known about the structural nature of the subunits within the complex. The majority of research on insect olfaction has been carried out either in vivo or in vitro utilising downstream indicators such as change in fluorescence (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013) or electrical current (Jones et al. 2011; Pask et al. 2011; Chen and Luetje 2012; Jones et al. 2012). This PhD project was undertaken to; firstly develop a protocol for the expression and purification of the subunits, and secondly to apply a classical protein biochemistry approach to investigate OR subunit interactions, and thirdly to investigate the crystallisation conditions for the OR subunits to undertake further X-ray crystallography experiments.

6.1.1 Expression and Purification of the Insect OR subunits

At the commencement of this PhD in 2010 there were no reported attempts to overexpress and purify the insect OR subunits in the literature. Therefore, an empirical approach had to be taken to evaluate the optimum expression system and buffer conditions to overexpress and purify these receptors. Five different expression systems were evaluated for their ability to overexpress six insect OR subunits. Two prokaryotic systems were unable to produce the receptors even when codon optimised; however, three eukaryotic systems were capable of producing all subunits tested with varying levels of expression (Chapters 2 and 3).

To assess their ability to solubilise the insect OR subunits from cell membranes or the wheat germ cell-free system, a panel of detergents representing the three classes (ionic, nonionic and zwitterionic) were screened. This screening experiment demonstrated that the subunits produced in the cell-free system were able to be solubilised in a greater number of detergents than the subunits produced from the baculovirus-mediated Sf9 cells. However, the wheat germ cell-free system is not cost-effective to scale up to the quantities needed to purify receptors for many subsequent biochemical approaches (e.g. SEC-MALS or crystal
trials). The DmOrco subunit could be solubilised in a larger number of detergents than the DmOrX subunits when expressed using baculovirus in Sf9 cells. However, of the 19 detergents screened, only one non-denaturing detergent, Zwittergent 3-16, was able to solubilise all the receptors tested in each expression system (Table 2.3). This detergent was subsequently used to maintain the receptors in a soluble environment for further experiments.

A two step purification protocol was established using affinity tag immobilisation of His$_{10}$-tagged subunits on Ni-NTA followed by size exclusion chromatography (SEC). The purification schedule required an extensive detergent screen to optimise the solubilisation conditions. It is interesting to note that several detergents that have proven useful in solubilising membrane proteins from the literature were not capable of solubilising the insect ORs, which proves the importance of carrying out an empirical approach to optimising purification conditions for membrane proteins. This protocol successfully purified all subunits tested with the exception of DmOr35a which, for unknown reasons, could not be expressed with a His$_{10}$ tag.

Three purified receptors; DmOR10a, DmOr22a and DmOrco, generated monodisperse peaks on the SEC profile, albeit with varying amounts of aggregation. The other two receptors resulted in either a double peak (DmOR43b) or a heterogeneous profile of predominantly aggregated protein (DmOr67d) (Figure 3-3). Purified DmOr22a and DmOrco were assessed for structural integrity using circular dichroism spectroscopy and found to be highly structured, containing >70% α helix with some β sheets. This provided evidence that the receptors were potentially folded correctly and could thus be used in further experiments to resolve their stoichiometry and structure.

### 6.1.2 Analysis of the oligomeric structure of the purified insect OR subunits

The insect OR complex is thought to be a ligand-gated non-selective cation channel and as such could be comprised of more than one subunit forming either homo- or heteromeric complexes (outlined in section 3.1.1). The Orco subunit is capable of forming an ion channel when expressed alone in heterologous cells (Jones et al. 2011; Pask et al. 2011; Chen and
Luetje 2012; Jones et al. 2012), and is therefore potentially forming a homomeric ion channel \textit{in vivo} that is activated by an OrX subunit. If these homomeric and heteromeric complexes are stable they should be able to be purified intact, or reconstituted from purified subunits.

Initial Native PAGE analysis indicated that the purified DmOrX and DmOrco subunits are capable of forming higher order homomeric structures indicating potential dimers, trimers, tetramers, and hexadecamers. To investigate the stoichiometry of the subunits from the Native PAGE gels, these potentially oligomeric bands could be excised and subjected to mass spectrometry analysis (Zheng et al. 2011; Chen et al. 2013). However, the heteromeric OR complex could not be purified intact and so no analysis of the stoichiometry of the subunits involved could be carried out. When the individual OR subunits were analysed using SEC-MALS it was apparent that under these conditions the majority of the protein was monomeric with the potential to form dimers. However, the SPR experiments provided preliminary evidence that DmOr22a and DmOrco can interact on the chip surface, though these experiments will need to be repeated and expanded to test for non-specific binding events. If the results of these experiments hold true with controls, SPR will be a useful method for investigating the structure and function of insect ORs, as it provides a more direct form of evidence for this interaction.

\textbf{6.1.3 Analysis of the Interactions between receptor subunits}

There is evidence from the literature that an interaction between the subunits is occurring both \textit{in vivo} and \textit{in vitro} (Neuhaus et al. 2005; Benton et al. 2006; Tsitoura et al. 2010; Pask et al. 2011; German et al. 2013), and the presence of Orco is essential to ensure correct trafficking of the OrX subunits to the dendritic membrane in \textit{Drosophila melanogaster} (Benton et al. 2006). Experimental evidence also demonstrates that both the Orco and OrX subunits affect the structure of the ion channel indicating that they likely interact in the membrane to form a receptor complex and perhaps cooperate in the formation of the pore (Pask et al. 2011). These data point to the receptors forming a stable complex of at least two subunits. I used affinity-tag based pull-down assay in combination with cross-linking to investigate this interaction between the receptor subunits.
Pull-down assays did not provide any evidence for an interaction between either the DmOrX subunits and DmOrco, or any of the DmOrX-DmOrX or DmOrco-DmOrco combinations tested. This result is likely due to the buffer conditions and choice of detergents, as an interaction between Orco and mosquito OrX subunits (Tsitoura et al. 2010) has proven the utility of this assay. However, there are some points of difference between their experimental approach and mine. Tsitoura et al. (2010) used 2% DDM to solubilise the insect OR subunits expressed in a Bombyx mori cell line, and incubated the resulting lysate from the His\textsubscript{10}-tagged subunit with Ni-NTA slurry. In contrast I carried out a membrane isolation step and detergent solubilised the membrane bound OR subunits prior to loading the His\textsubscript{10} tagged subunit on Ni-NTA resin. I then washed the resin extensively prior to loading the non-His\textsubscript{10} tagged subunit and again washed extensively prior to eluting the protein. However, Tsitoura et al. (2010) did not wash the resin prior to adding the non-His\textsubscript{10} tagged subunit nor did they elute the protein from the resin prior to analysis SDS PAGE. There could be an endogenous factor (such as lipids or unknown proteins) in the cell lysate that aids the interaction between the subunits which is retained in solution in the Tsitoura et al. (2010) protocol. These endogenous factors could be stripped away following the more stringent solubilisation and wash strategy that I used. These endogenous factors could play an important role in maintaining the structure of the OR subunits which allows them to interact, or the endogenous factors themselves could be interacting and providing a false positive for the OR interaction. This difference in protocols should be investigated in more detail to further examine the potential for pull-down assays to be used in elucidating an interaction between insect OR subunits.

Although this thesis aimed to use a protein biochemistry approach to investigate the structure of the insect OR complex, it was apparent that inherent characteristics of this method could impair the ability of the receptor subunits to interact \textit{in vitro}. To overcome some of these problems the stoichiometry of the receptors in HEK 293 cell membranes was investigated with a single molecule fluorescence photobleaching approach. Single molecule fluorescent photobleaching experiments have been successfully used to unravel the stoichiometry of many membrane proteins including the insect ionotropic glutamate receptors (Abuin et al. 2011). We had previously demonstrated with FRET analyses that
insect OR homomeric and heteromeric complexes can be detected in heterologous cell membranes when tagged with fluorescent proteins (German et al. 2013). Four *D. melanogaster* insect ORs were successfully tagged with either GFP or mCherry fluorescent proteins and visualised in lysed HEK 293 cell membranes immobilised on cover slips. However, the single molecule photobleaching experiments proved unsuccessful. This result might be due to several points of difference with my approach compared to that taken in the literature. The majority of reported experiments have used total internal reflection fluorescence (TIRF) microscopy to analyse a thin layer within 100 nm of the cell surface, which allows an analysis of the whole cells without the need to lyse them. I did not have access to a microscope with this capability and so followed the protocol by Hallworth et al. (2012) who were able to detect single molecules of the membrane protein prestin in lysed HEK 293 cell membranes. Additionally, the microscope lens used in my approach had much lower resolving power than those used in reported experiments. This difference in lenses could have a major effect on the ability to resolve single molecules in the membranes. This line of investigation remains open to potential collaboration with researchers who have a microscope with the required specifications.

Conclusions concerning the *in vivo* characteristics of the receptor subunits should not be made solely from the results of *in vitro* experiments such as those carried out in this study. It is very difficult to make conclusions based on negative results and further experiments are required to provide evidence for a potential interaction. However, I would note that further work using a protein biochemistry approach to investigate the interactions between insect ORs should focus on SPR experiments rather than pull-down assays. There are many inherent factors in these experiments that could modify the behaviour of the receptors, from the expression system chosen to the choice of buffer and detergent, and the temperature at which the experiments are carried out. Therefore, the lack of evidence for a heteromeric complex in this study should not be taken as direct evidence that the receptors do not form a heteromeric ion channel *in vivo*. The behaviour of recombinantly expressed protein can also be drastically different than that found *in vivo*. This was recently demonstrated by Richard Benton’s lab when they found that the LUSH mutants previously shown to diminish or enhance spontaneous and CVA evoked activity when recombinantly
expressed and infused into olfactory sensilla (Laughlin et al. 2008) did not have an effect on pheromone response when expressed transgenically (Gomez-Diaz et al. 2013).

I have demonstrated that both DmOrco and DmOr10a are functional when expressed in HEK 293 cells and yet there is no evidence from the pull-down assays that these two subunits are interacting. The addition of cross-linking reagents was expected to stabilise the functional DmOrco/DmOr10a complex in HEK 293 cells and allow an interaction to be observed. However, cross-linking did not show a pull-down interaction between these subunits when expressed in either HEK 293 cells or Sf9 cells. The OrX subunits have been demonstrated in the literature to remain functional in HEK 293 cells without the presence of Orco (Nakagawa et al. 2005; Grosse-Wilde et al. 2006), as they are interacting with a stably expressed Gα protein that can activate secondary signalling cascades to open ion channels. This secondary messenger mechanism might still be the major mode of function when the DmOrX and DmOrco subunits are expressed together in HEK 293 cells. There is therefore the possibility that a stable OrX/Orco heteromeric complex is not required to enable odorant reception. Activation of the Orco ion channel might result from the secondary messengers activated by ligand-bound OrX subunits interacting with endogenous Gα proteins (Atwood et al. 2011). If this is the case, the two OR subunits might not be directly interacting in this expression system and thus a pull-down or cross-linking experiment would not elucidate the structure of this receptor complex.

6.1.4 Screening for Conditions to initiate Crystal Formation

As of September 2013, no structures of insect odorant receptors have been reported. Current structural knowledge is based on in silico predictions from the primary amino acid sequence, and epitope tagging experiments in heterologous cells (Benton et al. 2006; Smart et al. 2008; Tsitoura et al. 2010). These experiments indicate that the receptors are seven transmembrane domain proteins similar in structure to GPCRs but with an intracellular N-terminus and extracellular C-terminus. The exact position of the TM domains and loop regions is unclear; however the Orco subunit has a significantly larger intracellular loop 2 than the OrX subunits, potentially indicating a gating mechanism for the ion channel. The related family of potassium channels share many characteristics with insect ORs including a
potential ion selectivity filter in DmOrco (Benton et al. 2006). Potassium channels are obligate tetramers made up of either homomers or heteromers. Computational modelling has indicated that insect ORs are capable of forming both homo- and heteromeric ion channels (Figure 6-1) when a potassium channel is used as a template (Harini and Sowdhamini 2012). However, this has not been confirmed with experimental evidence, and the loop domains were not included in the model.

Figure 6-1: Image of selected DmOrco transmembrane regions in a tetrameric form superimposed on the Potassium channel KCSA. Computational modelling of DmOrco indicates the potential for it to form a tetramer similar to the potassium channels. Modified from (Harini and Sowdhamini 2012).

Generating a high resolution structure of an insect odorant receptor would be of great benefit to the field. It would allow the testing of hypotheses around channel formation and function, ligand-binding and protein-protein interactions, as well as the rational design of agonists and antagonists. Chapter 5 outlined the initial conditions that have provided crystals of the DmOrco subunit and the expansion of that initial condition which also
resulted in crystal formation. The other four DmOrX receptors that were able to be purified were also screened with 192 conditions, although there were no crystals formed as of September 2013. These screens are still being monitored for crystal formation. There are plans to expand both the purification and the screening conditions to include more detergents, stabilising reagents and scaffold proteins, which might enable production of more crystals for the OR subunits.

6.2 Future work

The majority of research on insect olfaction has taken a genetics based approach using the excellent *Drosophila melanogaster* model organism, or expression in heterologous cell systems. A protein biochemistry approach however, allows for direct analysis of the interactions between OR subunits without the need for downstream indicators such as behavioural changes in the fly or changes in fluorescence in cell-based assays.

The first step for carrying out protein biochemistry experiments is identifying expression systems that can successfully produce the protein of interest. At the time that the purification protocol was developed I did not have access to the inducible HEK 293 system. Initial expression trials conducted in a non-inducible HEK 293 cell line demonstrated that the level of protein produced was too low to attempt large scale purification trials. It is possible that more protein would be expressed in the inducible system that could be amenable to solubilisation and large-scale purification; however this remains to be tested. Further work also needs to be carried out to identify conditions that will allow the purification of the intact functional OR complex for investigation into its structure, and the stoichiometry of the subunits involved.

One of the most important components for solubilising membrane proteins and maintaining them in solution is the choice of detergent (Garavito and Ferguson-Miller 2001). There are a number of detergents that have been shown to solubilise membrane proteins in the literature, many of which were not tested in this study. Expanding the initial detergent screen to include a wider range of detergents might uncover more conditions for the solubilisation of these receptor subunits and potential complexes, and increase their
stability in solution. Exchanging DmOrco into DDM provided evidence for potential dimer formation by SEC-MALS, but it remained monomeric in Brij 35, and aggregated in sodium cholate. This detergent screen should also be expanded to identify all the detergents that maintain the subunits in a soluble form. This might identify a detergent that cannot be used to solubilise the OR subunits from cell membranes, but which could promote oligomeric states after exchange.

Detergents are not exact mimics of the cellular membrane environment, and the degree to which a detergent is able to provide a suitable replica of the bilayer depends on the particular protein and detergent (Postis et al. 2008). There is substantial interest in the development of novel membrane mimics that can be targeted to the solubilisation and stability of membrane proteins. This has led to the development of synthetic polymers known as amphiphiles which have been used with success to both solubilise membrane proteins, and maintain their structure and function in solution (Park et al. 2007; Dahmane et al. 2009; Chae et al. 2010; Park et al. 2011). The research into polymers is regularly producing new amphiphiles that can be tested for their suitability to insect OR research, and provides another avenue for future work to establish optimum conditions for their purification and storage.

Future work on the purification of these receptors should focus on increasing the stability of the subunits in solution. Ensuring the receptors remain stable in solution is imperative when investigating their structure and function as any instability issues could affect their interaction with other subunits. In Chapter 5 several methods, such as the addition of ligands and stabilising compounds, were outlined that could be used to increase the stability of membrane proteins, and how these can be assessed in a relatively rapid manner using Florescence Size Exclusion Chromatography (FSEC), and heat FSEC.

Although this thesis provided only preliminary evidence for an interaction between the insect OR subunits, there is sufficient evidence from the literature to assume that some form of interaction does take place in vivo. The site for this interaction between OR subunits is thought to be the third intracellular loop based on yeast-2-hybrid analyses (Benton et al. 2006). To further investigate the site for this interaction, mutagenesis of the
predicted regions could be carried out. A protein biochemistry approach including SPR and SEC-MALS will allow the rapid analysis of these mutants and their affect on protein-protein interactions and ligand binding.

If the insect ORs are forming heteromeric ion channels similar to potassium channels, the differential signalling of particular OrX-Orco complexes might be due to the fact that each complex could comprise a different ion-conducting pore composed of domains from both the Orco and OrX subunits. This pore could confer differential selectivity to ions, thus altering the signalling capability of the complex. The affect of mutations on the potential ion selectivity region could also be examined using a protein biochemistry approach. However, in order to examine the ion-conducting properties of the complex in a purified form a functional assay will need to be developed. The development of a functional assay for these receptors would also provide some confidence that they are folding correctly and interacting to form a complex. Though no such assay exists at present for isolated receptors, initial experiments using proteoliposomes formed by merging DmOrco-expressing HEK 293 membranes with preformed liposomes demonstrate that a Planar Lipid Bilayer system could be used as a method to test these receptors for a response to their respective ligands. Future work should include further development of this assay or one similar to provide evidence that the purified OR subunits are folding correctly and forming a functional complex. Other potential functional assays for purified protein include ion flux assays using radioactive isotopes (Nimigean 2006), or liposomes preloaded with fluorescent dye (Pielak et al. 2009).

### 6.2.1 Application of insect ORs to biosensors and Pest management

The ability to overexpress and purify insect OR subunits in a form that is predominantly monomeric, while maintaining some degree of secondary structure is an important step in the implementation of insect receptors in biosensing devices.

Current electronic sensors (e-noses) are generally based on an array of metal oxide sensors that can respond to a range of organic compounds (Berna et al. 2009). There are a number of other technologies that are commercially available but have not been accepted for widespread use due to poor performance in some real-world tasks (Berna et al. 2008; Röck
et al. 2008). Insect ORs could potentially provide exquisite sensing capabilities as they have a broader odorant specificity than metal oxide sensors (Berna et al. 2009) and can detect odorants at concentrations as low as a single molecule. The next step in the development of insect ORs as a biosensor would be to immobilise them in a manner that allows the ligand-binding event to be reported and monitored in real time. I have demonstrated that immobilising insect OR subunits on SPR chips maintains their ability to interact with other OR subunits, this could be expanded to examine their ability to interact with ligands on the chip surface. However, SPR is not amenable to miniaturisation, which would be necessary to use it as a biosensor in real world tasks, and thus would be more useful as a diagnostic tool to examine immobilisation strategies for other platforms.

Mammalian ORs have been successfully incorporated into biosensor formats and shown to transduce signals associated with ligand binding (Dacres et al. 2011; Goldsmith et al. 2011), however, GPCRs such as the mammalian ORs undergo a known conformational change when a ligand is bound, which can used as the basis for a response. Insect ORs are not GPCRs and have not been demonstrated to undergo a conformational change upon ligand binding, and as such this approach might not work. However, the first step in designing a potential insect-OR biosensor would be to investigate the potential for a conformational change upon ligand binding solely to the OrX subunit. If this approach is unsuccessful there is the possibility of using the ion channel capabilities of the Orco subunit alone or the OrX/Orco complex to transduce a signal upon ligand binding (Krishnamurthy et al. 2010; Krishnamurthy et al. 2010).

Insects are major horticultural pests that can cause widespread agricultural damage which can have a severe economic impact (Oliveira et al. 2013), they are also vectors for many diseases including malaria and dengue fever. Developing a greater understanding of the insect OR complex will allow for the generation of a new class of insect repellents and pest management strategies. Generation of a structure of insect ORs and in particular the exact ligand-binding site will allow the rational design of new molecules which can be used in pest management to specifically target individual species (Rinker et al. 2012; Taylor et al. 2012). This will also allow the generation of high throughput assays (such as the ion flux assays) to identify and evaluate new OR blocking agents that directly affect the receptors. This has the
potential to drastically change the way insect pests are currently dealt with in the field by reducing the amount of broad spectrum pesticides and generating new ‘cleaner’ and more directed alternatives.
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