BIOCHEMICAL AND BIOPHYSICAL CHARACTERISATION OF ANOPHELES GAMBIAE NADPH-CYTOCHROME P450 REDUCTASE

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PHILIP WIDDOWSON

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ABSTRACT

As the principal vector for the transmission of the *Plasmodium falciparum* parasite, and hence the spread of malaria in Sub-Saharan Africa, *Anopheles gambiae* is a globally significant species of mosquito. Over recent years, the efficacy of established insecticides has waned and there is a constant need for novel effective compounds. Cytochrome P450 reductase (CPR) is a diflavoprotein known to have a central role in phase I metabolism of xenobiotic compounds and, in mosquitoes, this involves the detoxification of insecticides. Due to an inherent lack of understanding regarding the mechanisms of action of *A. gambiae* CPR, the selective inhibition of this enzyme is a previously untried approach. This project aims to biochemically characterise *A. gambiae* CPR in direct comparison to the human enzyme.

It was found that *A. gambiae* CPR was deficient in bound FMN, and to a lesser extent FAD, relative to human CPR with 20 % less FMN bound to the purified mosquito protein. Following the dissection of *A. gambiae* CPR into its constituent FMN- and FAD-binding domains, and using Isothermal Titration Calorimetry (ITC), a 4-fold decreased was observed for the binding affinity for FMN in the *A. gambiae* FMN-binding domain as compared to the equivalent human protein. The redox potential of the oxidised/semiquinone transition of the *A. gambiae* FMN-binding domain was -92 mV, much more negative than the published value for human FMN-binding domain. These data suggest a clear difference between these enzymes in the binding strength of FMN and its propensity to accept electrons.

The binding characteristics of NADPH nucleotides were probed in some detail. Comparison of the binding of NAD$^+$ and NADP$^+$ revealed a strong bias for the phosphate containing NADP$^+$. In addition, the position of the phosphate was important as 3’-AMP bound very poorly whilst 2’-AMP bound more strongly. 2’, 5’-ADP binding highlighted the importance of additional stabilising interactions involving the 5’-phosphate. Comparison of 2’, 5’-ADP and NADP$^+$ binding confirmed that the 2’-phosphate interaction was the principal site for NADPH recognition and provided the majority of the binding energy for this interaction. *A. gambiae* CPR was shown to bind NADPH nucleotide analogues 2’-AMP, 2’, 5’-ADP and NADP$^+$ much less strongly than the human enzyme highlighting a potentially significant difference in coenzyme binding. Binding affinities for the nucleotide ligand to intact CPR and the isolated FAD domain showed that the FAD-binding site is fully contained within the FAD-binding domain.
differences in the thermodynamic parameters between the intact enzyme and the isolated FAD-binding domain suggest that, although not directly involved in NADPH binding, the presence of the FMN binding domain had an effect on the overall binding energetics.

Despite an apparent difference between *A. gambiae* and human CPR in flavin incorporation and NADPH binding affinity, it was interesting that the activity of cytochrome *c* reduction of both enzymes was similar. The measured $K_m$ with respect to NADPH corroborated the ITC data by suggesting a stronger interaction of the coenzyme with human CPR compared to *A. gambiae* CPR. There was an approximate 2-fold increase in potassium ferricyanide reduction with the isolated *A. gambiae* FAD-binding domain compared to the intact enzyme with the presence of the FMN-binding domain again seemingly imparting an effect of events involving the FAD-binding domain.

In order to fully understand and rationalise all of the data, a comprehensive structural determination of *A. gambiae* CPR is required. With this in mind, isotopic labelling and subsequent biophysical analysis was carried out on the intact CPR and its FMN- and FAD-binding domains. Successful labelling was achieved for all samples, including the deuteration of the intact CPR and FAD-binding domain. However, the greatest success involved the FMN-binding domain with sufficient triple resonance spectra collected for backbone assignments. Although this success could not be matched for the intact CPR and FAD-binding domain, the work has provided a solid base for more a comprehensive study in the future.
ABBREVIATIONS

ACT – artemisinin combination therapy  
AChE – acetyl cholinesterase  
2’, 5’-ADP – adenosine 2’, 5’-diphosphate  
AEX – anion exchange chromatography  
2’-AMP – adenosine 2’-monophosphate  
3’-AMP – adenosine 3’-monophosphate  
BES – N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid  
BM3 – Bacillus megaterium P450 reductase  
BSA – bovine serum albumin  
CPR – NADPH-cytochrome P450 reductase  
CYP – cytochrome P450  
DDT – di-chloro di-phenyl trichloroethane  
DNase1 – deoxyribonuclease  
dNTPs - deoxyribonucleotides  
DTT - dithiothreitol  

E° - redox potential  
EDTA – ethylenediaminetetraacetic acid  
FAD – flavin adenine dinucleotide  
FADH₂ – FAD semiquinone  
FADH₂ – FAD hydroquinone  
FMN – flavin mononucleotide  
FMNH₂ – FMN semiquinone  
FMNH₂ – FMN hydroquinone  
FNR – ferredoxin NADP⁺-reductase  
GST – glutathione S-transferase  
H₄NADP – 1,4,5,6-tetrahydro-NADP  
His-tag – 6x histidine tag  
HSQC – heteronuclear single quantum coherence  
HPLC – high performance liquid chromatography  
IPTG – isopropyl β-D-1 thiogalactopyranoside
ITC – isothermal titration calorimetry

kDa – kilodaltons

$K_d$ – dissociation constant

LB – Luria Bertani medium

MHz - megahertz

MOPS – 3-(N-morpholino)propanesulfonic acid

MSR – methionine synthase reductase

NAD$^+$ - reduced nicotinamide adenine dinucleotide

NADH – nicotinamide adenine dinucleotide

NADP$^+$ - reduced nicotinamide adenine dinucleotide phosphate

NADPH – nicotinamide adenine dinucleotide phosphate

NMN – nicotinamide moiety

NMR – nuclear magnetic resonance

NOS – nitric oxide synthase

NR1 – novel reductase 1

OD$_{600}$ – optical density at 600 nm

PABA – $p$-aminobenzoic acid

PCR – polymerase chain reaction

PDB – protein data bank

PMSF – phenylmethanesulfonylfluoride

p.p.m – parts per million

RO H$_2$O – reverse osmosis H$_2$O

ROS – reactive oxygen species

r.p.m – revolutions per minute

SDS – sodium dodecyl sulphate

SERCA – sarco/endoplasmic reticulum-Ca$^{2+}$ATPase

SR – sulfite reductase

TEMED – N,N,N,N$^+$-tetramethylethylenediamine

TEV – tobacco etch virus

TROSY – transverse relaxation optimised spectroscopy
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Chapter 1

1. INTRODUCTION

1.1 Mosquitoes

Mosquitoes are common insects found in many different environments throughout the world. Although they are more prevalent in tropical climates some, more robust species can also be found in much cooler, temperate regions. They are a globally significant member of the class Insectera and the number of known species is in the region of 3,500. Mosquitoes are probably most renowned as vectors for some of the most major infectious diseases with more than 60 species being responsible for the transmission of such infections. Yellow Fever and Dengue Fever are transmitted by Aedes species such as Aedes aegypti whilst Anopheline mosquitoes such as Anopheles gambiae are responsible for malarial transmission in humans. It is perhaps worth noting that Anopheline mosquitoes are the sole vectors for human malarial transmission. Whilst both male and female mosquitoes feed on nectar, the female mosquitoes are capable of hematophagy by
Fig. 1.1. The life cycle of the *Anopheline* mosquito. The four stages of development are shown along with the average time it takes to progress to the next stage. The length of time spent at each stage will differ depending on species as well as environmental factors such as temperature.
administering bites to mammals; this is the reason for the spread of mosquito-borne infectious diseases.

Mosquitoes undergo a four-stage life cycle from egg to adult via two intermediate stages; larva and pupa (Figure 1.1). Eggs are laid on water and providing the temperature is adequate, hatch within 2-3 days. The larvae rest just below the surface of the water and 1-2 weeks later then develop into pupae. After 2-4 days, pupae metamorphose into fully developed adult mosquitoes. Adult Anopheline mosquitoes have a typical life-span of anywhere between one week and one month.

1.2 Malaria

Malaria is a vector-borne disease of major global significance responsible for up to and beyond 3 million deaths per year worldwide. The highest prevalence for the disease is in the Sub-Saharan African region where up to 90% of incidences of clinical illness are thought to occur (Breman et al., 2004) as well as the vast majority of deaths in children under five (Snow et al 2005). Malaria is caused by Plasmodia parasites which are transmitted to humans through the bite of Anopheline species of mosquitoes. There are four species of Plasmodia which promote human infection: Plasmodium malariae, Plasmodium
Clinical symptoms of malaria, chills and fever, along with more severe complications such as hypoglycaemia, anaemia and coma are attributed to invasion and development within human erythrocytes during the *Plasmodium* life cycle (Weatherall *et al*., 2002). The full life cycle takes place within both mosquito and human hosts and begins when *Plasmodium* sporozoites are transferred to the human bloodstream following a blood-meal from an infected *A. gambiae* female (Figure 1.2). Sporozoites circulate in the bloodstream for a short time before the invasion of hepatocytes. For *P. falciparum* the time between transfer of sporozoites and their subsequent invasion of hepatocytes is a matter of minutes. Within 10-12 days, infected hepatocytes rupture releasing thousands of daughter merozoites which invade circulating erythrocytes and over the next 48 hr they embark upon the clinically important intra-erythrocytic cycle of asexual replication. The action of *P. falciparum* differs from other malarial parasites in that parasitized erythrocytes do not remain in circulating blood for the entirety of their lives. Within 24-32 hr of invasion by merozoites, young parasites mature into trophozoites and the parasitized erythrocytes adhere to endothelial cells or to various bodily organs. At this stage of development, the trophozoites mature further
one of two ways. They either mature into schizonts which rupture and release daughter merozoites to go on and invade fresh erythrocytes. This pattern of invasion, release and re-invasion into erythrocytes highlights the cyclical pattern of clinical pathologies associated with malarial infection (Weatherall et al. 2002). Some trophozoites however will mature into male and female gametocytes. These are taken back into the mosquito when a second A. gambiae female takes a meal. Once inside the mosquito, the gametocytes fuse and undergo full development into sporozoites before being transferred to the salivary glands ready for re-admission into a fresh human host (Haldar et al 2007).

1.3 Treatment of malaria

Targeting infective Plasmodia parasites is imperative in the treatment of malarial symptoms and there are currently a large number of compounds available.

1.3.1 Chloroquine

Chloroquine (Figure 1.4) is one of the more traditional malarial treatments and its mode of action is to interfere with the parasitic heme
Fig 1.2. The life cycle of the *Plasmodium falciparum* parasite. The parasites require both mosquito and human hosts in order to fully develop into fully infective entities. The major symptoms of malaria occur during the invasion of the erythrocytes by *P. falciparum* merozoites (Adapted from Haldar et al. 2007)
detoxification processes (Krugliak and Ginsburg, 1991; Bray et al., 1998). In red-blood cells, parasites need to degrade haemoglobin to acquire essential amino acids. Heme, however, is toxic to parasites and thus is bio-crystallised to form to non-toxic molecule hemozoin. Chloroquine inhabits parasitic cells by diffusion and caps hemozoin preventing further crystallisation, thus, causing an accumulation of heme in the parasitic food vacuole. Chloroquine then binds to heme resulting in an extremely toxic chloroquine-heme complex which causes parasitic cell lysis and subsequent autodigestion (Hempelmann, 2007). Chloroquine, therefore, has no effect against sporozoites or exo-erythrocytic stages of parasites which do not consume haemoglobin.

One major problem however is the growing levels of resistance to chloroquine in *P. falciparum* rendering it almost useless as a treatment. Resistance is due to mutations in transporters resulting in decreased chloroquine concentration at the parasitic food vacuole, its site of action. Chloroquine is, however, still effective against infections with *P. vivax, P. ovale* or *P. malariae* (Deen et al., 2008). Due to chloroquine resistance, alternative aminoquinolines amodiaquine and primaquine (Figure 1.4) are more readily used. Amodiaquine works in a similar manner to chloroquine and has some efficacy against low-level chloroquine resistant *P. falciparum* strains (Olliaro et al., 1996). However, due to cross-resistance, it is completely ineffective in places
with higher levels of chloroquine resistance (Lemnge et al., 2006; Nsimba et al., 2008). The mechanism of action of primaquine is unknown but is effective against all intra-hepatic forms of malarial parasite (Deen et al., 2008).

1.3.2 Sulfadoxine

Sulfadoxine (Figure 1.4) is a slowly eliminated sulfonamide and acts as a competitive inhibitor of the enzyme dihydropteroate synthase. Folic acid is essential for DNA and RNA synthesis in many species and \( p \)-aminobenzoic acid (PABA) is an intermediate of folic acid synthesis. PABA is non-essential in humans as they lack the necessary enzymes to convert it into folic acid. Sulfadoxine is structurally similar to PABA and, thus, interferes with the activity of dihydropteroate synthase which is responsible for the conversion of PABA to folic acid meaning the parasitic growth is limited through folic acid deficiency whilst having no effect on humans (Deen et al., 2008).
1.3.3 Pyrimethamine

Pyrimethamine (Figure 1.4) is a diaminopyrimidine which acts by inhibiting plasmodial dihydrofolate reductase and consequently blocking the synthesis of nucleic acids within the parasites. As in the case of dihydropteroate synthase, dihydrofolate reductase is similarly involved in the conversion of PABA to folic acid.

Dihydrofolate reductase is an enzyme that catalyses the reduction of dihydrofolate to tetrahydrofolate using NADPH as an electron donor (Figure 1.3 A). Dihydrofolate reductase has been recently studied on a biophysical level and a number of solution structures have been solved in complex with NADPH and various inhibitors (Kovalevskaya et al., 2005; Beierlein et al., 2009)

Whilst sulfadoxine has no effect on humans, pyrimethamine can lead to folic acid deficiency in humans due to effects on host dihydrofolate reductase. It is effective against all forms of human malarial causing Plasmodia; however, resistance is developing. Currently, pyrimethamine is no longer used in isolation; it is only used in combination with a sulfonamide, traditionally sulfadoxine. As a combination, sulfadoxine and pyrimethamine maximise efficacy by acting on two separate enzymes in the conversion of PABA to folic acid (Figure 1.3 B) (Deen et al., 2008).
Figure 1.3. The role of dihydrofolate reductase and dihydropteroate synthase in tetrahydrofolate metabolism. 

A = The mechanism of tetrahydrofolate metabolism by dihydrofolate reductase. NADPH bound to dihydrofolate reductase is the electron donor in the reduction of dihydrofolate to tetrahydrofolate.

B = The positions of dihydropteroate synthase and dihydrofolate reductase in the conversion of PABA to tetrahydrofolate.
The sulfadoxine-pyrimethamine combination has rapidly become the successor to chloroquine in places of high-level resistance. Unfortunately, resistance to the sulfadoxine-pyrimethamine combination is growing in *P. vivax* but in areas where *P. vivax* is still susceptible, this combination is still a widely used treatment (Hawkins et al., 2007). Sulfadoxine-pyrimethamine is appropriate as the unified malarial treatment in places where *P. falciparum* and *P. vivax* coexist and are both susceptible to this combination. The numbers of areas which meet these criteria are rapidly declining and there is a growing need for alternative treatments in these and other malaria hot-spots (Leslie et al., 2007).

1.3.4 Quinine

Quinine is another traditional compound for the treatment of malaria. It is an alkaloid (Figure 1.4) derived from the bark of the *Cinchona* tree. Its proposed mode of action involves, similar to chloroquine, the inhibition of the heme detoxification in the food vacuole; however, its mechanism is poorly understood. It is effective against all stages of *P. vivax*, *P. malariae* and *P. ovale* but is ineffective against mature gametocytes of *P. falciparum*. Acyl aminoalcohols mefloquine, halofantrine and lumefantrine are all relatives of quinine but are rarely
given alone. They are commonly given as drug combination therapies involving artemisinin derivatives (Deen et al., 2008).

1.3.5 Artemisinin derivatives and artemisinin-based combination therapies (ACTs)

Artemisinin (Figure 1.4) is extracted from the leaves of the sweet wormwood (Artemisia annua) It acts rapidly as a blood schizontocide against all malarial Plasmodia parasites. Its broad activity means it is active against all stages along the parasitic life cycle including against P. falciparum gametocytes which are otherwise only sensitive to primaquine (Pukrittayakamee et al., 2004). There is no consensus regarding the precise mode of activity of artemisinin. On a chemical level, artemisinin is thought to bind to liberated heme, produced following haemoglobin degradation, leading to the generation of reactive oxygen species (ROS) and subsequent cell death (Cumming et al., 1997). There is evidence that artemisinin inhibits cysteine protease activity within the digestive vacuole leading to haemoglobin accumulation and inhibition of hemozoin (Pandey et al., 1999). Alternatively, it has been suggested that artemisinin, acts via inhibition of the essential calcium adenosine triphosphatase (SERCA) activity (Eckstein-Ludwig et al., 2003).
Artemether, artemotil and artesunate are the three major derivatives of artemisinin and in vivo become converted to dihydroartemisinin, the very potent active metabolite. Today, artemether, artemotil and artesunate are very effectively combined in artemisinin-based combination therapies (ACTs) with other compounds and the current recommended combinations for treatment of malaria, particularly *P. falciparum* derived, are: i) Artemether-lumefantrine, ii) Artesunate and amodiaquine, iii) Artesunate and mefloquine, iv) Artesunate and sulfadoxine-pyrimethamine (Deen et al., 2008).

The advantages of ACT lie primarily in overcoming the effects of drug resistance. The use of a second compound will render the therapy effective even if there is growing resistance to one of the partners. Another key advantage is that artemisinin and its derivatives are remarkably safe and extremely well-tolerated by humans making them ideal as treatments (Ribeiro and Olliaro, 1998; Price et al., 1999). The major considerations when contemplating use of ACTs are cost and availability as well as levels of resistance to the partner compound in the area of interest. Deciding on the correct therapy can be difficult and is complicated further in areas of vast multidrug resistance. Worryingly, however, *P. falciparum* resistance to artemisinin has been shown to be emerging in isolated areas of South-East Asia which, if it spreads further afield, could have major consequences for current
malarial treatment strategies (Dondorp et al., 2009) particularly as currently, there are no compounds currently in-line to replace artemisinin should resistance become more widespread (Dondorp et al., 2010).
Figure 1.4. The chemical structures of some of the major compounds used in malarial treatments. The vast diversity of compounds is displayed and a wide variety of compounds is shown from the more traditional chloroquine and quinine to the more modern used artemisinin.
1.4 Insecticide use

The use of pesticides, principally to protect crops, has been common place for many hundreds of years. Sulphur-based compounds, as well as toxic chemicals such as lead, mercury and most commonly arsenic were the main weapon against invading pests. This was the case until the discovery of the insecticidal properties of dichlorodiphenyltrichloroethylene (DDT) in 1939 by chemist Paul Müller (Turusov et al., 2002).

Insecticides, pesticides used to target insects, are used in a multitude of ways. They are used to protect to crops and plants, both agriculturally and in house-hold gardens; however, in many regions of the world, such as Sub-Saharan Africa, their major use is in the prevention of spread of vector-borne disease such as Malaria. However, the high intensity and prolonged usage of such insecticides is having a dramatic impact on a whole host of ecosystems (Relyea et al., 2005).
1.4.1 Organochlorines

Dichlorodiphenyltrichloroethane (DDT) (Figure 1.5) is probably the most notable of the organochlorine class of insecticides. Its mode of activity involves maintaining Na\(^+\) channels in an open state. With the Na\(^+\) channels unable to close, the dramatic alteration of polarity leads to severe convulsions followed by death of the insect (Narahashi, 1992). DDT is very efficiently stored in all tissues of living organisms and is highly fat soluble. The compound itself is not acutely toxic but due to its poor biodegradability and heavy, prolonged usage, DDT has become concentrated and accumulated within food-chains. The higher up the food-chain, the more severe the problems have become with pronounced effects on avian populations. There are cases of marked thinning of eggshells within populations of birds such as the falcon (Turusov et al., 2002) as well as reproductive failing in other birds such as grebes, ospreys and eagles (Fry, 1995). These ecological issues have led to the subsequent banning of DDT and other related compounds in a number of countries.
Figure 1.5. Structural examples of the four major groups of chemical insecticides. The structures shown are of the organochlorine, DDT; the organophosphate, malathion; the carbamates, carbofuran and aldicarb and the pyrethroid, permethrin.
1.4.2 Organophosphates

Organophosphate insecticides, such as malathion (Figure 1.5) and parathion, have similar effects on mammals as they do on insect which is down to their mode of activity. They target, and phosphorylate, serine hydroxyl groups on the enzyme acetylcholinesterase (AChE). With the AChE activity impaired, there is an accumulation of the key neurotransmitter acetylcholine at cholinergic synapses. This build-up of acetylcholine is the cause of a number a symptoms and death is generally due to respiratory failure. The modification of AChE by phosphorylation is potentially irreversible depending on the chemical structure of the organophosphate and rate of dephosphorylation. If left phosphorylated for long enough, the AChE can be considered irreversibly inhibited and the only means of restoring function is by synthesis of fresh enzyme (Costa, 2006).

The analogous nature of insect AChE compared to its mammalian counterparts makes these compounds as toxic to humans as they are to the insects they are meant to target. However, they hold some advantages over other insecticides such as DDT. The major advantages are that they are highly biodegradable and thus do not accumulate within ecosystems, unlike the organochlorines. This coupled with their high efficacy as well as low cost, has made the likes of malathion and parathion ideal insecticides (Fukato, 1990).
1.4.3 Carbamates

The carbamate insecticides have a mechanism of action that is virtually identical to the organophosphates. They also target the AChE enzyme. The only difference is that whilst organophosphates impair AChE activity by phosphorylation of serine hydroxyl groups, the carbamates target the same serine hydroxyl groups by addition of a carbamyl group. This carbamylation is more readily reversible than the phosphorylation by organophosphates and a simple hydrolysis reaction can easily restore nerve function. Despite this, the carbamates are still regarded as potent inhibitors of AChE. Two common carbamate insecticides are carbofuran and aldicarb (Figure 1.5) (Fukato, 1990).

1.4.4 Pyrethroids

The environmental and human health issues associated with the organophosphates, organochlorines and carbamates has accelerated the need for a ‘safer’ class of insecticides. Pyrethrin is a natural organic product contained within the seed cases of the pyrethrum plant (Chrysanthemum cinerariaefolium) and analysis of its structural properties led to the production of the first synthetic pyrethroid – allethrin.
Other pyrethroids, such as permethrin (Figure 1.5) and deltamethrin, have subsequently been synthesized and are becoming the first choice insecticide across most parts of the world. Pyrethroids are similar in their mode of action to the organochlorines in the sense that they target the insect Na\(^+\) channels leading to a series of convulsions, closely followed by death (Vijverberg and van den Bercken, 1990). There are two major advantages of the pyrethroids over other insecticides. Firstly, they are effective against a much broader spectrum of pests than other insecticides. Secondly, they lack efficacy towards humans. Mammalian Na\(^+\) channels are in the region of 1,000 times less sensitive to pyrethroids than the insect Na\(^+\) channels and this, coupled with a higher body temperature and faster metabolism of the compounds in mammals, makes pyrethroids ideal pest-control agents (Vais et al., 2001).

1.5 Insecticide resistance

Initially, the use of DDT and other such insecticides and their effect on mosquito populations was remarkably successful. This early success in the control of malarial-vectors gave hope that the disease could be eradicated. However, the increase in disease prevalence over recent years has clearly indicated that this hope was false. The principal
reason for this is the pronounced increase in insecticide resistance (Ranson et al., 2000a). Although the basis of some of this resistance is still poorly understood, it is clear that insecticide resistance generally occurs either through enzymic metabolism of the compounds (Hemingway and Ranson, 2000) or through insensitivity of the target site, rendering it resistant to insecticide action. A specific example of the latter is the observed mutation in a Na\(^+\) channel gene in pyrethroid resistant *Anopheles gambiae* strains (Ranson et al., 2000b).

Three classes of enzymes have been shown to confer resistance to insecticides; the carboxylesterases, the glutathione-S-transferases (GSTs) and the monooxygenases. Insecticide resistance is conferred by either amplification of the genes coding for these enzymes, altered gene expression leading to an over-expression of the enzymes or enhanced metabolic activity of the enzymes (Hemingway et al., 2004).

1.5.1 Esterase-mediated insecticide resistance

Esterase enzymes have been extensively studied as mediators of insecticide resistance. The majority of the work regarding the role of esterases in resistance has been performed on *Culex* mosquitoes and the *Myzus* aphid (Hemingway and Ranson, 2000) although other species such as *Anopheles* mosquitoes are also known to show
esterase-mediated resistance (Vulule et al., 1999). The increased levels of these carboxylesterases observed in resistant strains are generally the result of amplification of the genes coding for the enzymes resulting in increased enzyme synthesis (Mouches et al., 1986; Field et al., 1988). The mechanism of activity of these implicated carboxylesterases is perhaps a consequence of their overproduction in that their principal mode of action is by rapid binding and slow turnover of the insecticide. This leads to more of a sequestration of insecticide activity rather than an extensive metabolism of them (Kadous et al., 1983).

1.5.2 GST-mediated insecticide resistance

Glutathione-S-transferase (GST) is another enzyme implicated in conferring insecticide resistance. GST acts on insecticides by catalysing the nucleophilic attack of reduced glutathione (GSH) on electrophilic centres of lipophilic compounds (Hemingway and Ranson, 2000). Early studies suggested that the primary role of GST in insecticide resistance was by way of detoxification of DDT (Hemingway et al., 1985). As is the case of the carboxylesterases, many resistant insects have been shown to have elevated levels of GST (Grant et al., 1991), further enhancing the case for its role in
insecticide resistance. It would appear however that there are differences at the molecular level between how carboxylesterase and GST concentrations are increased. Whereas the increase in carboxylesterases is primarily down to gene amplification, GST levels are enhanced by alterations in regulatory genes leading to increased production and hence overall activity of these enzymes (Grant and Hammock, 1992).

1.5.3 Monooxygenase-mediated insecticide resistance

Due to the large number of implicated enzymes, monooxygenases confer the most common form of insecticide resistance to current compounds. Similarly to GST-mediated resistance mechanisms, the monooxygenases are generally up regulated as a result of alterations in regulatory genes (Cohen et al., 1994). Monooxygenases, such as cytochromes P450, are slightly different from other detoxification enzymes in that they are also required to activate certain organophosphate compounds to their more toxic form (Hemingway et al., 2004) and resistance can be conferred due to either a decrease in this activation as well as the conventional increase in detoxification (Scott et al., 1999).
Likewise with carboxylesterase and GST-mediated mechanisms, monooxygenases are also shown to be up-regulated in resistant strains (Vulule et al., 1994) but this system is complicated further by the requirement for binding partners to the monooxygenases. NADPH-cytochrome P450 reductase (CPR) is required by all cytochromes P450 to achieve its function and the up-regulation of CPR has also been implicated in pyrethroid resistance (Lycett et al., 2006). It is this binding partner, the CPR, which I shall focus on in more detail.

### 1.6 NADPH-cytochrome P450 reductase

NADPH-cytochrome P450 reductase (CPR) is a 78 kDa diflavin-containing enzyme found primarily bound to the membrane of liver microsomes within eukaryotic systems. CPR is also found in bacterial systems; however, the machinery is quite different in these prokaryotes with the *Bacillus megaterium* P450 reductase (P450BM3) being a prominent example (Narhi and Fulco, 1986). Eukaryotic CPRs have four distinct domains; the N-terminal FMN-binding domain, the linker region, the C-terminal FAD/NADPH-binding domain and connecting domain. The FMN and FAD/NADPH-binding domains interact, respectively, with the prosthetic groups flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These cofactors are essential...

1.6.1 The diflavin-reductase ‘family’

The domain organisation of CPR and other members of the diflavin reductase family are represented (Figure 1.6) and there is a marked similarity between them. Although the amino acid sequences differ slightly between the members of the family the overall domain structure is remarkably similar. Whilst novel reductase I (Paine et al., 2000) and methionine synthase reductase (Leclerc et al., 1998) are almost identical to CPR, they lack only the membrane anchoring region, other diflavin-reductases are more noticeably different in organisation.

The previously mentioned P450BM3 (Narhi and Fulco, 1986) is unique amongst diflavin-reductases in that its cytochrome P450 element is linked to the reductase element in a single polypeptide chain (Figure 1.6) effectively providing *B. megaterium* with a fully functioning monooxygenase system with only the need for a single protein. Nitric oxide synthase (NOS) is another member of the family with its own additional features (Bredt et al., 1991).
Fig. 1.6. A schematic overview of the domain organisation of the diflavin reductase family. In the above, CPR = cytochrome P450 reductase; NR1 = novel reductase; BM3 = B. megaterium P450 containing reductase, SR = sulfite reductase; NOS = nitric oxide synthase and MSR = methionine synthase reductase. FAD and NADPH bind at separate regions within the same FAD/NADPH-binding domain (orange). FAD binds towards the N-terminus of the domain whilst NADPH bids at the C-terminus. *The α-subunit in SR is one of a octomer containing 3 other α-subunits along with four heme containing β-subunits.
Unlike P450BM3, which is linked to a cytochrome P450, NOS has an N-terminal oxygenase domain. This catalytic domain is linked to the reductase domain by a calmodulin binding region. Sulfite reductase (SR) is the member of the family most different to the rest (Ostrowski et al., 1989). In the case of SR, the FMN- and FAD/NADPH-binding domains first come together to form the α-subunit. Four of these α-subunits then combine to form what is effectively an octomer containing 4 FMN and 4 FAD molecules. In the fully formed molecule, the α-subunit octomer combines with 4 heme containing β-subunits resulting in a $\alpha_8\beta_4$ oligomer.

1.6.2 Ancestry of diflavin-reductases

Despite structural and functional differences between the members of the diflavin-reductase family, the presence of the ‘reductase domain’ remains constant throughout. It is now commonly regarded that this linking of an FMN-binding region to an FAD/NADPH-binding region to create a single diflavin-reductase has come about through a genetic fusion event (Porter and Kasper, 1986). Indeed, studies have uncovered significant homology between the major domains of diflavin reductases, such as CPR, and the two bacterial, monoflavin electron transport enzymes. The FMN-binding domain of CPR shows a distinct
homology to the bacterial flavodoxin whilst the FAD/NADPH-binding domain is homologous to the ferredoxin-NADP⁺ reductase (Porter and Kasper, 1985). Evidence for CPR, and other diflavin-reductases, essentially being a fusion of these two bacterial enzymes comes from the fact that they themselves can serve as an electron transport system for cytochrome P450, albeit at a much lower rate (Jenkins and Waterman, 1998). The same is observed when the FMN- and FAD/NADPH-binding domains from CPR are expressed individually. The two domains can be recombined to regain reductase activity, again however at only a fraction of the rate of the intact enzyme (Smith et al., 1994). In both cases, this lower rate of activity is likely to come from the fact that in CPR, the inter domain linker region (Figure 1.6) acts to bring the flavin cofactors closer together so as to increase electron transfer efficiency (Sevrioukova et al., 1996).

1.6.3 Electron transfer in CPR

The transfer of electrons through CPR enzymes is a complex process and one that has been studied extensively. The coenzyme NADPH binds and delivers a pair of electrons in the form of a hydride ion to the FAD cofactor. In the majority of cases, the two-electron reduced FAD hydroquinone then supplies FMN one electron at a time before final
reduction of the terminal acceptor. There are two types of electron acceptor; firstly, those which accept electron from the FMN-binding domain such as cytochromes P450. Cytochrome c is a non-physiological one-electron acceptor which also interacts at the FMN-binding domain (Vermilion et al., 1981; Kurzban and Strobel, 1986). The second type, those which accept electrons from the FAD-binding domain such as ferricyanide. Oxidised nucleotide, NADP⁺, can also be an electron acceptor in a transhydrogenase reaction which is essentially a reversal of the hydride ion reduction of FAD (Murataliev et al., 2004).

For multi-substrate enzyme reactions, there are two schemes of kinetic mechanism: Ping-Pong and either ordered or random Bi-Bi (Cleland, 1977). The Ping-Pong kinetic scheme requires the substrates to bind one at a time; essentially, the first substrate binds and is released before the second substrate binds and has its effect. The Bi-Bi scheme differs in that both substrates bind at the same time to make a substrate-enzyme ternary complex (Figure 1.7). This complex acts as the reactive intermediate before the substrates disassociate (Cleland, 1977). In the case of insect CPR, early work suggested a Ping-Pong mechanism of kinetics for house fly (Musca domestica) (Wilson and Hodgson, 1971; Mayer and Prough, 1977) and the southern armyworm (Spodoptera eridania) (Crankshaw et al., 1979). However, more
recently, studies on primarily house fly CPR have provided compelling evidence that CPR actually operates with a random Bi-Bi kinetic mechanism (Murataliev et al., 1999).

In addition to uncovering the kinetic mechanism, work on house fly CPR has also identified the rate limiting step of enzyme activity, in this case using cytochrome c reduction. The results show that the rate of hydride transfer from NADPH to FAD is about half the rate of overall catalysis. These data strongly imply that the rate of hydride transfer, and subsequent FAD reduction, is the rate limiting step in enzyme catalysis (Murataliev et al., 1999). This is in agreement with an earlier finding that for rat liver CPR, interflavin electron transfer occurs at a much faster rate than FAD reduction by NADPH (Oprian and Coon, 1982).

Another important question when considering electron transfer in CPR is which species acts as the terminal electron donor: the FMN semiquinone species (FMNH·) or the FMN hydroquinone species (FMNH$_2$). There are two distinct forms of the FMN semiquinone CPR. The first form is generated following two electron reduction, after hydride donation, from NADPH. The second form is the so-called ‘air-stable’ semiquinone, so named presumably because of its remarkable
Figure 1.7. A representation of the types of kinetic mechanism for multi-substrate systems. A – Ping-Pong mechanism where only one substrate is ever bound at one time. B – Random Bi-Bi mechanism where both substrates bind concurrently before activity and subsequent product release. In this scheme the reaction can take place along either pathway with the same end result achieved. In the above, E=enzyme, A and B = substrates, P and Q = products. Figure re-created from Murataliev et al., 2004.
stability in atmospheric oxygen; it is formed over time following incubation with stoichiometric amounts of NADPH. Interestingly, of the two forms of FMN semiquinone, only the first form is catalytically active whereas the ‘air-stable’ form fails to effectively reduce cytochrome c. It is postulated that following prolonged incubation with nucleotide, the ‘air-stable’ FMN semiquinone drifts into a kinetically stabilised conformation rendering it catalytically inactive (Murataliev and Feyereisen, 1999).

The ability of CPR to readily form the ‘air-stable’ semiquinone has compromised study of the kinetic mechanism of the enzyme. Early studies highlighted the formation of the ‘air-stable’ semiquinone which was shown to be catalytically inactive. Because of this, the general assumption was that the FMN hydroquinone was the active species in enzyme catalysis (Masters et al., 1965). Two models were proposed where the enzyme cycled through 1-3-2-1 or 2-4-3-2 reduction states where the numbers signify the number of electrons present on the flavin cofactors. In both models, the FMN hydroquinone was the electron donor and a priming reaction was required for the enzyme to achieve a 3 or 4 electron state. During more recent studies, a number of observations have highlighted inconsistencies and contradictions in both the 1-3-2-1 and 2-4-3-2 cycles (Murataliev et al., 2004).
The observations, mentioned above can be summarised as follows. Firstly, although house fly reductase is able to oxidise two molecules of NADPH, but because NADPH will only readily bind to fully oxidised CPR, only oxidation of the first molecule is rapid enough to be considered part of the catalytic cycle. This strongly implies that no priming reaction is required for rapid catalysis (Murataliev and Feyereisen, 1999). Secondly, the 2-4-3-2 reduction state cycle should be excluded as a possible mechanism based on the unfavourability of its formation. Four-electron reduced CPR can only be achieved following approximately 8 hr under anaerobic conditions in the presence of both NADPH and a coenzyme regeneration system (Vermilion and Coon, 1978).

With the 2-4-3-2 cycle all but eliminated as a possibility, the focus is turned to whether the 1-3-2-1 cycle is a probable kinetic mechanism or not. If this cycle is realistic as a potential mechanism, for one molecule of NADPH to reduce two cytochrome c, at least one of the electron pair must be delivered from the FMN semiquinone form meaning the semiquinone is a catalytically active species (Murataliev and Feyereisen, 1999). During steady state catalysis, only a minor proportion of CPR is in the FMN hydroquinone form (Murataliev and Feyereisen, 2000). This coupled with the fact that the rate of FMN hydroquinone formation from the semiquinone is around 5-10 times
slower than the maximal rate of cytochrome c reduction (Oprian and Coon, 1982; Shen et al., 1989; Bhattacharyya et al., 1991) strongly points towards the FMN semiquinone as the terminal electron donor. Although the above comparisons are between experiments performed on house fly, rabbit and rat CPR’s, the same can be seen in human CPR where the rate of hydroquinone formation is considerably slower than cytochrome c reduction (Gutierrez et al., 2001). Although cytochrome c, the terminal acceptor used for catalysis experiments, is a non-physiological terminal acceptor, comparable results can be seen when cytochrome P450 is incorporated (Guengerich and Johnson, 1997).

Other members of the diflavin reductase family provide compelling evidence for the electron transfer mechanism is these enzymes; P450BM3 has been extensively studied and in this enzyme, the two-electron reduced form is the catalytically active species. The three-electron reduced form is catalytically inactive and, as in CPR, the four-electron reduced enzyme is unobtainable in aerobic conditions (Li et al., 1991; Murataliev et al., 1997). The FMN semiquinone in sulfite reductase is also catalytically active (Zeghouf et al., 1998).

All these data combined had led to the proposed catalytic mechanism in house fly CPR. NADPH binds to the enzyme and transfers two electrons, in the form of a hydride ion, to FAD. Two-electron reduced FAD transfers one electron to FMN to form an FAD/FMN double
A. NADPH $\Leftrightarrow$ NADP$^+$ + H$^+$ + 2e$^-$

B. Cytochrome c (Fe$^{3+}$) + e$^-$ $\Leftrightarrow$ Cytochrome c (Fe$^{2+}$)

Figure 1.8. The proposed catalytic mechanism of cytochrome c reduction in *Musca domestica* CPR. A = The oxidation of NADPH which occurs during stage 2 of the cycle generates two electrons (2 e$^-$) and a proton (H$^+$). B = The reduction of cytochrome c which occurs during stages 4 and 6 of the cycle. Two different cytochrome c molecules bind to and accept and electron from CPR. Electrons are passed to the cytochrome c molecules one at a time which gives rise to the proposed catalytic cycle of 0-2-1-0. Flavin cofactors become fully reduced (FADH$_2$) by accepting two electrons or partially reduced (FAD- or FMN-) by accepting one electron. Figure adapted from Murataliev *et al.*, 2004.
semiquinone species. This form is the catalytically active electron donor and once the FMN delivers an electron, the FMN semiquinone is replenished with the second electron from FAD. In essence the catalytic mechanism acts with a 0-2-1-0 reduction state cycle with electrons donated from the FMN semiquinone form (Murataliev and Feyereisen, 1999). An overview of the proposed mechanism of electron transfer is detailed in Figure 1.8.

1.6.4. Interacting partners of CPR

CPR is a key component of many pathways and thus has the ability to bind to and affect a number of interacting partners. These binding partners include squalene monooxygenase, heme oxygenase, fatty acid desaturase, cytochrome b₅ and cytochromes P450. Interactions with the latter are possibly what CPR is best known for. However judging by the known interactions, it is clear that CPR is involved in a wide variety of different biological processes, enhancing its importance to cellular activity.
1.6.4.1 Cholesterol synthesis

Squalene monooxygenase, formally squalene epoxidase, is an essential enzyme in the process of cholesterol synthesis. It is involved during the later stages of the process and is responsible for the epoxidation of squalene to 2, 3-oxidosqualene prior to its conversion to lanosterol. It is a 64 kDa FAD-containing enzyme bound to the endoplasmic reticulum and requires interaction with CPR to act as a reductant following binding to NADPH (Laden et al., 2000).

1.6.4.2 Cytochrome b₅ reduction

Cytochromes b₅ are ubiquitous electron transport hemoproteins found in a variety of organisms. Microsomal and mitochondrial cytochromes b₅ are membrane bound whilst those found in erythrocytes are water soluble. Cytochrome b₅ becomes reduced by NADH via cytochrome b₅ reductase, an FAD-containing flavoenzyme, however by binding at the FAD-binding domain of CPR; cytochrome b₅ is also reduced by electrons acquired from NADPH (Enoch and Strittmatter, 1979). By acting through cytochrome b₅, CPR has since been implicated in fatty acid elongation by incorporation of malonyl-CoA into fatty acid chains (Ilan et al., 1981).
1.6.4.3 Lipid peroxidation

Lipid peroxidation is the oxidative degradation of unsaturated lipids by a free-radical chain reaction. It very readily affects cell membrane polyunsaturated lipids and can lead to damage of the membrane. CPR can act as the electron source for two stages of the lipid peroxidation reaction: the reduction of ADP-Fe$^{3+}$ to the radical ADP-Fe$^{3+}$-O$_2^-$ and also the formation of the lipid hydroperoxide species which itself creates new lipid radicals capable of sustaining the peroxidation process (Sevanian et al., 1990).

1.6.4.4 Heme oxygenase

Heme oxygenase is a microsomal enzyme required to catalyse the oxidation of heme, at the α-methene bridge, to form biliverdin and carbon monoxide. Biliverdin is subsequently converted, by biliverdin reductase, to bilirubin. NADPH and O$_2$ are required for oxidation of heme by heme oxygenase and it has become apparent that active heme oxygenase utilizes electrons gained from CPR to fulfil its function. Heme oxygenase is probably most active in the spleen in the conversion of heme from old erythrocytes (Schacter et al., 1972).
1.6.4.5 Cytochrome P450

The major physiological electron acceptors for CPR are the cytochromes P450. Cytochromes P450 (CYPs) are an extremely large super-family of heme-containing enzymes consisting of many hundreds of members and are found in nearly all forms of life. Eukaryotic CYPs are located within the membrane of the endoplasmic reticulum or mitochondria. They are heavily involved in the metabolism of fatty acids, steroids and, possibly most significantly, xenobiotic compounds using molecular oxygen as well as reducing equivalents, gained from CPR upon binding of NADPH (Feyereisen, 1999).

Insects come into contact with a multitude of exogenous compounds on a daily basis. CYPs, therefore play an essential role in the metabolism of such compounds. Insects contain hundreds of different CYP isoforms each with its own duty in the effective clearance of potentially toxic substances, indeed some compounds are actually activated by CYPs which could be either helpful or harmful to the insect. Figure 1.9 provides a simplified overview of the monooxygenase system involving CPR and CYP with CPR in a central position. The role of insect CPR in reducing CYP is vital in xenobiotic metabolism and subsequent conferring of monooxygenase-mediated insecticide resistance mechanisms. With this in mind, the potential for
Fig. 1.9. A schematic overview of the monooxygenase system involving cytochrome P450 and cytochrome P450 reductase. CPR, after interaction with coenzyme NADPH, reduces CYP as part of phase I metabolism in xenobiotic processing. CPR may also interact with a number of other acceptors such as heme oxygenase and squalene monooxygenase as well as a number of non-physiological acceptors including cytochrome c and potassium ferricyanide.
inhibition of CPR in insecticide resistant strains in particular, the Anopheline malarial vectors, cannot be ignored.

1.6.5 Sequence alignments of CPRs

For many years, CPR’s from a multitude of organisms have been studied. Figure 1.10 is the alignment of the amino acid sequences of the most commonly studied CPR’s with a few additions. The Anopheline mosquito’s A. gambiae and A. minimus, the well characterised Musca domestica and the model organism D. melanogaster are samples from the insect family. As well as these insect enzymes, the mammalian Homo sapiens and Rattus norvegicus have been compared. The remaining enzymes in the alignment are from the Saccharomyces cerevisiae whose structure was recently solved, the FMN-binding domain-like flavodoxin, and the FAD-binding domain-like ferredoxin and for additional comparison methionine synthase reductase (MSR), another member of the diflavin reductase family (Figure 1.6).

The alignment confirms the high levels of similarity between all the sample CPRs with particularly high homology between the insect derived enzymes and, likewise, between the rat and human samples. The yeast enzyme is the least similar to any of the rest of the sample;
however, at around 30% similarity to the other CPRs there is still a clear sequence similarity. The flavodoxin aligns well to the FMN-binding domain of all the CPR’s as does the ferredoxin align with the FAD-binding domains corroborating the theory that diflavin reductases such as CPR derived from some sort of fusion between these monoflavin proteins (Porter and Kasper, 1986).

The highlighted regions on the alignment show a high level of homology between all the CPR’s for the particular residues known to be involved in FMN, FAD and NADPH binding. Subtle differences, however, are present such as residue Y456 in rat CPR. This residue is implicated in FAD binding and differences in the equivalent residues in the Anopheline samples (H459 in A. gambiae and S459 in A. minimus) could potentially have an impact on FAD binding in these enzymes. Such high levels of similarity between the CPRs, particularly in the key residues for flavin and coenzyme binding, do not account for the differences in observed characteristics. It is more likely that local conformational differences could be responsible. There is, therefore, a need for more in-depth structural analysis in order to explain these differences.
The key residues in FMN, FAD and NADPH binding are highlighted in yellow. Residue Y456 (rat numbering) and its equivalents are highlighted in blue. The membrane anchor region, FMN-binding domain, inter-domain linker region and FAD/NADPH-binding domain boundaries are shown. The alignment was generated using ClustalW.
1.6.6 Structure of CPR

The domain organisation of CPR involving the N-terminal FMN-binding domain, the linker region, the C-terminal FAD/NADPH-binding domain and connecting domain is shown in Figure 1.6. Although the FMN- and FAD/NADPH-binding domains have the ability to fold and behave independent of each other (Porter and Kasper, 1985), the other major domains of CPR such as the linker region and connecting domain are absolutely essential for a fully functioning reductase (Smith et al., 1994). The first crystal structure of a CPR enzyme was that of the *Rattus norvegicus* CPR (rCPR) in 1997 by Wang et al. The solved crystal structure (PDB: 1AMO) is a soluble, NADP$^+$ bound form of the enzyme crystallised without the 6 kDa hydrophobic membrane anchor domain. Whilst this soluble CPR lacks the ability to interact with membrane bound CYPs, (Anderson et al., 1994) it retains enzymatic activity as shown by its ability to transfer electrons to non-physiological acceptors such as cytochrome *c*.

The FMN-binding domain consists of five α-helices flanking a five-stranded parallel β-sheet with the FMN cofactor binding to the tip of the β-sheet at the C-terminal side. The FAD/NADPH-binding domain comprises two separate entities, the FAD-binding portion and the NADPH binding portion. The FAD binding portion is an anti-parallel β-barrel whilst the NADPH-binding portion is of similar structure to
the FMN-binding domain with a parallel β-sheet sandwiched by α-helices. The primarily α-helical connecting domain appears to almost split the FAD-binding portion and this domain, along with the disordered inter-domain loop region appear to act as a hinge to bring the FMN- and FAD/NADPH-binding domains together. This positions the FMN and FAD flavins within close proximity of each other to enhance electron transfer efficiency (Wang et al., 1997). More recently, a second structure has been solved, that of N-terminal truncated *Saccharomyces cerevisiae* CPR in 2006 by Lamb *et al.*

Although the two structures appear well conserved with the same general structure consisting of the FMN-binding, FAD/NADPH-binding and connecting domains along with the inter-domain linker region, there appear to be two major observable differences between the two proteins.

The crystallised yeast CPR (yCPR) appears to have a second FMN molecule incorporated into the structure. The second FMN binding site (the ‘FMN2 site’) is positioned at the interface between the FMN-binding domain and the connecting domain. The second FMN cofactor appears to be less tightly bound to the CPR as it is more exposed and highly accessible to the bulk solvent. However, analysis of the flavin content of the yCPR gives an equimolar FMN:FAD:CPR ratio
Figure 1.11. The solved structure of rat CPR. The crystal structure was reported by Wang et al., 1997. The FMN-binding domain (blue), FAD-binding domain (green) and connecting regions (red) are clearly shown. The flavin cofactors are shown as purple ball and stick models whilst the partially resolved NADP⁺ is shown as orange. This truncated soluble form is without the membrane anchor region. PDB – 1AMO.
suggesting that only one of the two FMN-binding sites is normally occupied. The apparent unnatural occupancy of both FMN-binding sites in the solved structure could be due to the large excess of FMN added to the sample prior to crystal growth. It is thought that a single FMN cofactor shuttles between the two FMN-binding sites during electron transfer in yCPR. It has recently been shown that this second FMN binding site, despite being exposed at the protein surface, is highly specific for FMN over other similar compounds including FAD (Ivanov et al., 2010).

A second observed difference between the solved yCPR and rCPR structures is the conformation of the FAD adenosine moiety. In yCPR, the FAD adenosine sits in a hydrophobic pocket formed between the connecting and FAD/NADPH-binding domains whilst in rCPR its position is slightly shifted (Figure 1.12). It is possible that the position of the FAD adenosine moiety creates hindrance and prevents FMN binding to the ‘FMN2’ site which is conserved in rCPR. This is a possible reason as to why only one FMN cofactor is observed in the rCPR solved structure compared to the two FMN cofactors in the yCPR equivalent (Lamb et al., 2006).

Although the rat and yeast CPR structures have been the only ones currently solved for wild type enzymes, a number of other structures have also been reported. The range of W677 mutant rat CPR’s
Figure 1.12. The relative orientation of the flavin cofactors in yeast and rat CPR’s. A = Yeast CPR, B = Rat CPR. In both cases the FMN (blue), FAD (red) and NADP⁺ (purple) are shown. The protein molecule has been removed in each case for ease of viewing. Note the presence of the second FMN molecule in yeast CPR. The FAD isoalloxazine ring and NADP⁺ in the rat CPR structure are poorly resolved. The different orientation of the FAD adenosine ring is highlighted. Figures generated using PyMol and PDB numbers 1AMO for rat CPR and 2BF4 for yeast CPR.
(Hubbard et al., 2001) are, like the rat and yeast structures, in a closed conformation with FMN and FAD in close proximity. However, recently, a mutated rat CPR with four amino acids deleted from the inter-domain linker region has been solved in three extended open conformations (Hamdane et al., 2009). In these structures, the distances between the FMN and FAD cofactors are between 29 and 60 Å rather than the 3.5 Å seen in the wild type rat CPR structure (Wang et al., 1997). It is suggested that CPR is likely to adopt such an open conformation when interacting with cytochrome P450. The structure of a yeast FMN-human FAD-domain chimera in an open conformation has also been solved showing a similarly large distance between the flavin cofactors with a separation of 86 Å (Aigrain et al., 2009).

1.6.7 FMN-binding domain

The N-terminal FMN-binding domain is primarily involved in interactions with its physiological partners, CYP molecules, and with non-physiological proteins such as cytochrome c. The position of the FMN cofactor means it is regularly exposed to the surrounding solvent, particularly when the domain moves via the hinge region during electron transfer. This exposure to solvent explains the findings that
FMN easily and readily dissociates from the CPR (Vermillion and Coon 1978).

In the solved rCPR structure, the FMN cofactor was shown to be bound to fragments $^{139}$TYEGPD and $^{175}$NKTYEHFN where tyrosine residues Y140 and Y178 cover the isoalloxazine ring of FMN on the re and si-sides respectively (Wang et al., 1997) (Figure 1.13). Prior to full structural determination, previous biochemical data highlighted the necessity of these residues. Sequence comparison of rat liver oxidoreductase with *Desulfovibrio vulgaris* flavodoxin indicated Y140 and Y178 were indeed involved in FMN-binding. Site-directed mutagenesis confirmed that substitution of Y178 with a non-aromatic aspartate virtually abolished both FMN-binding and subsequent catalytic activity. Although the same substitution of Y140 did not have a dramatic effect on FMN-binding, catalytic activity was compromised, suggesting that the cofactor binds in an unfavourable conformation for electron transfer (Shen et al., 1989).

Several flavodoxin structures from a wide range of species have been elucidated using both X-ray crystallography and Nuclear Magnetic Resonance (NMR) techniques. Flavodoxin structures from *Clostridia* (Burnett et al., 1974), *Desulfovibrio vulgaris* (Watt et al., 1991; Stockman et al., 1993; Knauf et al., 1996), the red alga *Chondrus crispus* (Fukuyama et al., 1992), *Anabaena* (Rao et al., 1992),
Azotobacter chroococcum (Peelen et al., 1996) and Desulfovibrio desulfuricans (Romero et al., 1996) have all been solved. In addition, the FMN-binding domain crystal structure of human CPR has also been solved by NMR (Zhao et al., 1999).

1.6.8 FAD/NADPH-binding domain

The larger C-terminal FAD/NADPH-binding domain is principally involved in binding NADPH thus beginning electron transfer through FAD and FMN to respective acceptor molecules. Unlike the FMN molecule, FAD is less readily available to the surrounding solvent due to its position within the CPR molecule. The isoalloxazine ring is positioned at the boundary between the FAD and NADPH binding sites; this is ideal positioning for accepting electrons from NADPH. The remainder of the FAD molecule is positioned at the hydrophobic interface between the FAD-binding and connecting domains.

In the solved rCPR structure, the FAD molecule is shown to be bound within the fragments 455YYSIAS and 471ICAVAV. The penultimate residue in the amino acid sequence, W677, is also essential in FAD binding as the tryptophan indole ring is stacked against the re-side of FAD whilst the residue Y456 stacks against the si-side of the flavin.isoalloxazine ring.
Figure 1.13. The residues involved in FMN and FAD-binding in rat CPR. A = the FMN binding site. The flavin is shown bound to the edge of the FMN-binding domain (blue) and is stacked by the two tyrosine residues. B = the FAD binding site. The key residues involved in FAD binding are highlighted including the W677 residue stacked against the isoalloxazine ring of FAD. In both cases for simplicity, some of the surrounding residues have been removed from the image. PDB-1AMO.
The pyrophosphate region is stabilised by the side chains of T491 and R454 and the ribityl-pyrophosphate chain lies almost parallel to residue C472 and Y478 (Figure 1.13). Y478 is further involved by stacking of its phenolic ring against the adenine ring of the adenosine moiety (Wang et al., 1997).

1.7 Nucleotide binding

NADPH binding is the key event in CPR catalytic activity, having a marked effect on a number of events including electron transfer and the rate of reduction on both physiological and non-physiological electron acceptors. Studies on the ferredoxin-NADP+ reductase (FNR) protein family have an established bipartite nature of coenzyme binding suggesting that the flexibility of the NADPH molecule is crucial in how it interacts with FNR as well as members of the diflavin reductase family such as CPR (Deng et al., 1999; Piubelli et al., 2000). A 2:1 nucleotide:protein ratio model indicating two NADPH binding sites had been proposed (Gutierrez et al., 2001); however, a more recent 1:1 stoichiometric model has been suggested (Daff et al., 2004). This 1:1 stoichiometry has since been confirmed and the 2:1 model withdrawn citing possible sample contamination with 2’-AMP.
following 2′5′-ADP sepharose purification, as the culprit for erroneous data (Grunau et al., 2006).

1.7.1 Recognition of 2′-phosphate

The bipartite nature of NADPH binding is further revealed by the presence of distinct binding sites for the 2′-phosphate and nicotinamide (NMN) moieties. The 2′-phosphate binding site in human CPR is shown to involve residues S596, R597, K602 and Y604 (Figure 1.14). Initial recognition of NADPH takes place at this 2′-phosphate binding site and interestingly, the NMN moiety seems to provide little by way of contribution to the overall binding energy. Probably the key residue in the 2′-phosphate binding site is R597 which is positioned to form a salt bridge with the 2′-phosphate of NADPH with the R597A mutant showing severely impaired nucleotide binding affinity. 2′-phosphate is the primary source of binding energy; however, the 5′-phosphate group has also been shown to be of significance. CPR has a much lower binding affinity for 2′-AMP than for 2′5′-ADP highlighting a synergistic effect involving 5′-phosphate. The residue likely to be involved in interacting with 5′-phosphate is R298 – a basic residue positioned close to the 2′-phosphate binding site. Recognition of the 2′-phosphate group is highly precise as shown by the very weak
Figure 1.14 The residues surrounding the 2’ phosphate binding site. Residues that make up the 2’ phosphate binding site are shown from the rat CPR structure. S596 (red), R597 (blue), K602 (cyan) and Y604 (purple) are shown surrounding the 2’ phosphate group of NADP⁺. PDB- 1AMO.
binding affinity of CPR for 3′5′-ADP in relation to 2′5′-ADP (Grunau et al., 2006).

CPR interacts with and receives electrons from NADPH much more effectively than NADH. The similarity in redox potentials observed when either nucleotide is used implies that electron transfer from NADH is just as energetically favourable as with the catalytic NADPH. Despite this, the maximal rate of catalysis is orders of magnitude faster when using NADPH (Shen et al., 1991; Murataliev et al., 1999). The principal reason for this efficient nucleotide discrimination is the much higher affinity which CPR shows for NADPH over NADH because of the presence of the 2′-phosphate moiety. Added to this is the fact that flavins reduced by an NADPH source are a lot more stable than those reduced by NADH (Dohr et al., 2001).

1.7.2 Reduction state effect on interaction

The highly flexible nature of NADPH not only affects the mechanism by which the nucleotide binds but also contributes to the shape of the molecule. In its reduced form, the NADPH adopts a flat conformation whereas the oxidised form adopts a so-called ‘boat’ conformation. The similarities in binding affinity of CPR to 2′,5′-ADP and NADP+
indicate a distinct lack of interaction between the oxidised NMN (NMN$^+$) moiety and oxidised flavoprotein. This is a different case for reduced NADPH meaning oxidised CPR binds oxidised and reduced nucleotides with different affinities. Addition of a positive charge, as well as the altered shape of the nicotinamide ring is the reason for this apparent specificity.

As well as the reduction state of the nucleotide, protein-nucleotide interaction is also dependent on the reduction state of the flavoprotein. Electron transfer and rapid enzyme catalysis occur when reduced NMN interacts with oxidised flavoprotein and as discussed above, the same oxidised enzyme cannot interact with NMN$^+$ moiety. In the same vein, reduced flavoprotein cannot accept further reducing equivalents from reduced NMN under normal aerobic conditions however it can interact with NMN$^+$. This is one of the more intriguing aspects of the activity of CPR activity as the enzyme can effectively undergo a reversal of traditional electron transfer. The transhydrogenase reaction occurs by transferring electrons, in hydride ion form, from FADH$_2$ to NADP$^+$ (Murataliev and Feyereisen, 2000). The difference in binding properties of protein of different reduction states is important to remember when ascertaining binding affinity and properties of NADPH. When establishing, for example, binding affinity an alternative form of NADPH must be employed. 1,4,5,6-tetrahydro
NADP (H₂NADP) is an example of a nonreactive analogue which has near identical binding properties of NADPH yet cannot serve as an electron donor and hence does not have the ability to alter the reduction state of the flavoprotein during the course of experiments (Grunau et al., 2006).

1.7.3 Structural aspects of nucleotide interaction

Structural study of this enzyme has brought to light some interesting features which could serve to explain mechanisms involved in nucleotide binding and the effect this may have on electron transfer and enzyme catalysis. Interaction of 2’-phosphate is integral in correctly positioning the NMN moiety for binding. Although the NMN portion of NADPH is not fully resolved in solved structures of CPR (Wang et al., 1997), FNR (Karplus et al., 1991) or sulfite reductase (Sibille et al., 2005) yet the position where it is expected to bind is occupied by a bulky aromatic residue. This added to the fact that the regions where the 2’-phosphate and nicotinamide moieties bind can be between 10-12 Å apart. These data suggest that, in order to overcome both the spatial overcrowding imparted by the aromatic residue and distance constraints, interaction with 2’-phosphate could be coupled with a conformational shift to allow fast hydride transfer from NADPH.
to FAD. This conformational shift is likely to be the reason for hydride transfer to FAD being the rate limiting step in catalysis (Muratalieev and Feyereisen 1999).

The residue C566 in rat CPR, equivalent residue C560 in house fly CPR (C568 in A. gambiae CPR), is close to where the NMN moiety is thought to interact. These suggestions are corroborated by evidence that the C560Y mutation shows decreased affinity for NADP⁺ but has little effect on 2’-AMP affinity. Other substitutions with less bulky residues serine and methionine failed to yield such an effect suggesting spatial hindrance involving the aromatic tyrosine and the nicotinamide ring (Murataliev et al., 1999). The rat CPR crystal structure places the C566 residue close to the adenine ribose of NADP⁺ so it is possible that the residue is brought to the nicotinamide binding site following initial NADPH interaction and subsequent conformational change (Wang et al., 1997).

1.7.4 Global effect of NADPH binding

NADPH binding, although occurring within the FAD-binding domain, is responsible for a number of effects throughout the enzyme. Not only is there a conformational change which allows NMN interaction, the structural shift has a more global effect. Inter-domain electron transfer
is strongly gated (Gutierrez et al., 2002) and binding of NADPH analogues leads to an increase of inter-domain electron transfer suggesting that FAD and FMN cofactors are brought into a favourable orientation for electron transfer (Gutierrez et al., 2003). Studies pertaining to the degree of separation of the two flavins show the degree of conformational shift that CPR undergoes following NADPH binding. In the ligand free protein there can be up to 20 Å separation of the flavins (Bastiaens et al., 1989) whilst the crystal structure, with NADP⁺ bound, positions the flavins as little as 4 Å apart (Wang et al., 1997).

Studies involving human CPR and the isolated FMN-binding domain with regards to different forms of cytochrome c reduction have highlighted the global effect that NADPH binding has on the rest of the enzyme. Fully oxidised CPR (or isolated FMN-binding domain) fails to interact with oxidised cytochrome c and the fact that reduced cytochrome c cannot interact with any form of CPR; this means that oxidised cytochrome c needs a form of reduced reductase to accept electrons.

Dithionite reduced FMN-binding domain gives rise to the FMN-semiquinone and subsequently, electron transfer and cytochrome c reduction take place. Despite the FMN-semiquinone being required for formation of the kinetically competent complex, the use of dithionite to
partially reduce CPR by one electron, thus leading to the formation of the ‘air-stable’ semiquinone, fails to show any binding or reduction with respect to cytochrome c. This resembles the inhibition seen when house fly CPR is subjected to prolonged incubation with NADPH to form the equivalent ‘air-stable’ semiquinone. The inactive ‘air-stable’ semiquinone becomes kinetically stabilised by drifting into a ‘locked’ conformation preventing interaction of the FMN-binding domain with cytochrome c (Murataliev and Feyereisen, 1999). Interestingly, only when CPR is reduced by NADPH does it form an interaction with cytochrome c which proves that the presence of a reduced species alone is not enough to lead to efficient electron transfer. The conformational change observed upon binding of NADPH to CPR effectively unlocks the FMN-binding domain into a position capable of cytochrome c interaction. The binding affinities of both full length CPR and the isolated FAD-binding domain to 2',5'-ADP are remarkably similar, thus placing the NADPH binding site totally within the FAD-binding domain. NADPH binding at the FAD-binding site causes not only a local conformation change but also has a more dramatic effect by impinging on events involving the FMN-binding domain, located at the opposite end of the enzyme to the NADPH-binding site (Grunau et al., 2006).
1.7.5 The W677 residue

The penultimate residue in all CPR sequences is a large, aromatic tryptophan (W677 in rat, W676 in human and W678 in A. gambiae CPR) (Figure 1.10). Analysis of a number of CPR, and related, structures locates this residue where the NMN of NADPH is expected to bind (Karplus et al., 1991; Wang et al., 1997; Ingelman et al., 1997; Gruez et al., 2000; Zhang et al., 2001). Due to its location, the residue must be displaced by the nicotinamide ring for electron transfer and catalysis to occur, as indicated by stopped flow fluorescence on human CPR (Gutierrez et al., 2001; Gutierrez et al., 2002). In addition to this, the solved structures of FNR (Deng et al., 1999) and CPR (Hubbard et al., 2001) mutants show NMN in a position which should allow for fast hydride ion donation. However, it is somewhat paradoxical that these mutants in fact have impaired electron transfer properties (Dohr et al., 2001; Elmore and Porter, 2002).

This diminished electron transfer ability could be explained when considering what is proposed to be the major role of this highly conserved residue. It is suggested that the residue is heavily involved in shielding of the FAD isoalloxazine ring from the surrounding solvent (Figure 1.15). When the residue is removed, the exposure to solvent is likely to have the effect of increasing the reduction potential of the FAD flavin (Calcaterra et al., 1995) making physiological
electron transfer to FMN thermodynamically unstable. If NADPH were then to be bound to CPR, the reduction potential of the FAD would be decreased, thus the favourability of electron transfer to FMN increased due to contact and shielding by the nicotinamide ring. This decrease in reduction potential is likely to impart some sort of energetic cost, such as increasing binding affinity for NADPH making release of the nucleotide more difficult. The proposed major role of this penultimate residue is therefore to shield the FAD cofactor, when the nicotinamide ring is not present, to limit the high energetic costs which are involved in decreasing the reduction potential to a level required for physiological activity (Murataliev et al., 2004).

Though this is the major proposed role for the penultimate tryptophan residue, other roles have been suggested such as being involved in discrimination between NADPH and NADH (Dohr et al., 2001), the removal of NADP$^+$ once hydride ion transfer has taken place (Gutierrez et al., 2000) and protection of the reduced FAD from re-oxidation by solvent once NADP$^+$ has been removed (Murataliev et al., 2004). The exact contribution that W677 provides is yet to be defined but the available evidence suggests it is likely that this important conserved residue has a number of duties within CPR and related enzymes.
Figure 1.15. The position of W677 residue in rat CPR. The position of the W677 residue in the rat crystal structure is clearly stacked against the isoalloxazine ring of FAD. The nicotinamide ring of NADP$^+$ is not resolved however it is thought to interact in the region where W677 occupies in the above structure. PDB – 1AMO.
1.8 Aims

The overall objective of this work is the biochemical characterisation of *A. gambiae* cytochrome P450 reductase and to make comparisons with the human enzyme. In addition, a biophysical study will be performed for the first time on *A. gambiae* CPR using NMR spectroscopy.

Specifically, the aims include:

a) The establishment of an *E. coli* expression system for efficient production and purification of recombinant *A. gambiae* and human CPRs. The design of expression vectors for the production and purification of the individual *A. gambiae* FMN-binding and FAD-binding domains for use throughout the project.

b) Characterisation of the spectral properties of the *A. gambiae* CPR and its domains including the establishment of the redox potentials of the intact reductase along with the isolated FMN-binding and FAD-binding domains. HPLC analysis will be used to determine the flavin contents of *A. gambiae* CPR and human CPR with a view to making a direct comparison between the two enzymes.

c) Stable isotopic labelling techniques using $^{15}$N, $^{13}$C and $^2$H isotopes will be investigated in order for NMR spectroscopy on *A. gambiae*
CPR, FMN- and FAD-binding domains to be performed. This will be the first instance of such biophysical analysis being carried out on A. gambiae CPR. The success of the NMR experiments will be determined by the efficiency of isotope labelling along with the stability of the protein samples.

d) Isothermal Titration Calorimetry (ITC) will be used as a tool for the probing of the NADPH binding site. NADPH nucleotide analogues will be utilised in the determination of binding and thermodynamic properties in the hope of establishing any potential differences that have arisen in coenzyme binding between A. gambiae and human CPR. ITC will also be used to compare the binding affinity of the flavin cofactor, FMN, to the isolated FMN-binding domains of A. gambiae and human CPR.

e) The activity of both A. gambiae and human CPR will be compared using the non-physiological electron acceptors cytochrome c and potassium ferricyanide. Kinetic parameters of cytochrome c reduction will be elucidated for each enzyme as a way of potentially increasing the level of understanding of A. gambiae CPR relative to the human equivalent.
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Water

Water was purified using a Direct-Q System (Millipore Corp., Mass. USA). This system removes ions, bacteria and organic material present in tap water through a reverse osmosis cartridge, followed by an ion-exchange resin cartridge. As a final purification step, the water is passed through a 0.22 µm filter as it is drawn from the system. The water produced has a resistivity of 18.2 MΩ cm or greater. This water was used for all procedures, excluding bacterial culture experiments. For molecular biology experiments nuclease free water (Sigma) was used.
2.1.2 General solvents

Ethanol: redistilled; Department of Chemistry, University of Liverpool.

All other solvents were of Analar grade from BDH Ltd., Poole, Dorset, UK unless otherwise stated.

All HPLC solvents were of HPLC grade and filtered and degassed prior to use.

2.1.3 General reagents

Unless otherwise stated, chemical reagents were supplied by Analar and were of laboratory grade. In general, reagents for molecular biological techniques were of high purity. BES buffer, 2'-AMP, 3'-AMP, 2',5'-ADP, NADP+ and NAD+ nucleotides for ITC experiments were supplied by Sigma. NADPH, horse heart cytochrome c, potassium ferricyanide, FMN and FAD were also all supplied by Sigma.
2.1.4 Expression vectors

**pET-15b**

**pETM-11**
2.1.5 Bacterial strains

BL21-CodonPlus(DE3)-RP strain:

*Genotype - E. coli B F− ompT hsdS(rB− mB−) dcm+ Tet4 gal λ(DE3) endA Hte [argU proLCam+]*

2.2 General Methods

2.2.1 Molecular Biology Methods

2.2.1.1 Polymerase Chain Reaction

Forward and reverse PCR primers were designed for molecular dissection of the full length *A. gambiae* CPR into its individual FMN- and FAD-binding domains. Primers were designed for cloning into the pETM-11 expression vector using the Kpn1 and Nco1 restriction enzyme sites. The PCR mixture was prepared in PCR tubes with special care taken to ensure that all components were kept on ice at all times. The PCR mixture contained the following, at final concentrations; 1x PCR buffer, 0.2 mM dNTPs, 1.5 mM MgSO₄, 1.2 μM forward primer, 1.2 μM reverse primer, 0.5 μL *A. gambiae* CPR template DNA. The mixture was made up to 49 μL using MilliQ H₂O before addition of 1 μL hot start *Kod* high fidelity polymerase
(Novagen). Control tubes were set up which contained no template DNA, all other components remained the same. All tubes were transferred to a thermo cycler for PCR.

2.2.1.2 Agarose gel electrophoresis

1 % (w/v) agarose gels were prepared as follows: 1 g agarose was dissolved in 100 mL 1 x TE buffer (1 L 10 x TE buffer consists 880 mL, 100 mL 1 M Tris; pH 8.0, 20 mL 0.5 M EDTA; pH 8.0). The mixture was heated until the agarose was fully dissolved and left to cool slightly. Once the mixture had cooled, but not sufficiently to allow the agarose to set, it was poured into a gel casting tray. Ethidium bromide to a final concentration 0.1 µg/mL was added and loading wells were formed using gel combs. The gel was left to stand for approximately 20 min to set before the combs were removed. 6 x sample buffer (sterile distilled water, glycerol, 0.25 % (w/v) bromophenol blue, 0.5 M EDTA) was added to each sample before being loaded into the wells. 1 kb standard markers were also loaded (New England Biolabs) before the gel was run at 120 V in 1 x TE buffer until the size of the bands could be determined relative to the markers; typically this took 20-30 min. DNA bands were visualised under U.V conditions using an ultraviolet transilluminator.
2.2.1.3 Restriction digestion of PCR products

Following PCR amplification, the DNA coding for FMN- and FAD-binding domains underwent restriction digestion. Care was taken to ensure all components remained on ice at all times. The restriction digestion mixture contained the following, at final concentrations; 1x restriction digestion buffer, 1x BSA, 15 µL DNA. The mixture was made up to a volume of 24 µL using MilliQ H$_2$O before addition of 3 µL Kpn1 (New England Biolabs) and 3 µL Nco1 (New England Biolabs). Another reaction was set up to digest the pETM-11 vector and was treated in exactly the same way as the PCR product. Reactions were allowed to proceed at 37 °C for 2 hr before being returned to ice. The reactions were stopped by addition of 6 x sample buffer and run on an agarose gel as described earlier (method 2.2.1.2). The visualised bands, both insert and vector, were carefully cut out of the gel and the DNA extracted using a gel extraction kit (Qiagen).

2.2.1.4 Ligation into expression plasmid

The digested inserts were ligated into digested pETM-11 vectors (Arie Geerlof, EMBL) using a quick DNA-ligase reaction. Care was taken to ensure that all components remained on ice throughout. A number of reactions were performed, each differing in the ratio of insert to vector;
a 1:1 insert to vector, a 2:1 insert to vector and a control reaction containing just the vector. The ligation reaction mixture for the 1:1 insert to vector contained the following: 1 x ligation buffer, 0.5 µL digested vector, 0.5 µL digested insert. The mixture was made up to 19 µL using MilliQ H₂O before 1 µL quick ligase (New England Biolabs) was added. For the 2:1 insert to vector reaction, 0.5 µL digested vector was incubated with 1 µL digested insert whilst the control simply contained 0.5 µL digested vector. The rest of the mixture remained the same. The ligation reaction was allowed to proceed at room temperature for 15 min before being returned to the ice. The mixture was then used to transform OmniMax cells (Source Bioscience LifeSciences, Nottingham) and allowed to grow on agar plates selective for the antibiotic kanamycin (see method 2.2.2.2)

2.2.1.5 Plasmid preparation from ligation product

Bacterial colonies were screened for the correct ligated plasmid by PCR. The PCR reaction was performed as above (method 2.2.1.1) using portion of the bacterial colony as the template DNA. The remaining portions of the colonies containing the correct insert were used to inoculate 10 mL LB media (Melford) containing 32 µg/mL kanamycin (Sigma). This was incubated overnight at 37 °C with
shaking at 170 r.p.m and the resulting culture used for plasmid preparation using a microcentrifuge mini prep kit (Qiagen). All new plasmids were sent for sequencing (Source Bioscience LifeSciences, Nottingham) to ensure correct incorporation of the insert.

2.2.1.6 Preparation of competent cells using the rubidium chloride method

1 µL of a commercial stock of BL21-CodonPlus(DE3)-RP E. coli was added to 50 µL selective LB media containing chloramphenicol (34 µg/mL) (Duchefa Biochemie) and left to grow overnight at 37 °C. 500 µL of this culture was used to inoculate a further 250 mL chloramphenicol-selective LB media containing 20 mM MgSO₄. This was returned to 37 °C shaking incubator and allowed to grow until an OD₆₀₀ = 0.4-0.6 was achieved. The cells were harvested by centrifugation at 10,000 x g for 5 min at 4 °C and resuspended in 100 mL ice-cold, filter-sterilized TFB1 (30 mM KAc, 10 mM CaCl₂, 50mM MnCl₂, 100 mM RbCl (Sigma), 15 % glycerol; adjusted to pH 5.8 using 1M acetic acid). The suspensions were incubated on ice for 5 min before once again being harvested by centrifugation at 10,000 x g for 5 min at 4 °C. The pellets were re-suspended in 10 mL ice cold, filter sterilised TFB2 (10 mM MOPS; pH 6.5, 75 mM CaCl₂, 10 mM
RbCl (Sigma), 15 % glycerol; adjusted to pH 6.5 using 1 M KOH). The suspensions were incubated on ice for 1 hr before being divided into 200 µL aliquots and flash frozen using liquid nitrogen. The transformation efficiency was determined prior to use (see method 2.2.1.7).

2.2.1.7 Transformation of competent E. coli

50 µL competent E. coli cells were thawed on ice and incubated with 2 µL plasmid DNA for a further 30 min on ice. The cells were heat-shocked at 42 °C for 45 s before being returned to ice for a further 2 min. 950 µL of non-selective LB media was added before incubation at 37 °C with shaking at 200 r.p.m for 1 hr. 100 µL of this culture was spread thinly on an agar plate; selective for either ampicillin (Melford) or kanamycin (Sigma), depending on the plasmid used, as well as chloramphenicol resistance and the plates incubated at 37 °C for approximately 16 hr. Control plates were also set up; one without antibiotic selectivity and another where no plasmid DNA was used in the transformation reaction.
2.2.2 Protein Expression Methods

2.2.2.1 *A. gambiae* CPR, *human* CPR and *human* FMN-binding domain expression in LB media

50 mL selective LB media containing ampicillin (250 µg/mL) and chloramphenicol (34 µg/mL) was inoculated with a single transformed bacterial colony containing the desired expression vector. This was incubated at 37 °C, with shaking at 170 r.p.m, for approximately 16 hr. This starter culture was centrifuged at 8,000 x g for 10 min at 4 °C and the pellet re-suspended in 5 mL fresh selective LB. Using this re-suspension, a further 1 L selective LB broth containing 1 mM riboflavin (Sigma) was inoculated to an OD$_{600}$ = 0.05-0.1 and divided equally into 4 x 2 L flasks. These were transferred to a 37 °C incubator and allowed to grow, with shaking at 170 r.p.m., until an OD$_{600}$ = 0.7-0.9 was reached. Typically, this took approximately 2-2.5 hr. All OD$_{600}$ measurements were taken using a Cary 300 Bio U.V visible spectrophotometer and the machine zeroed using fresh LB broth. Once the desired growth was achieved, protein expression was induced by addition of 1 mM IPTG (Sigma). The cultures were transferred to a 25 °C incubator, shaking at 170 r.p.m, for 16 hr. The bacterial cells were harvested by centrifugation at 8,000 x g for 10 min at 4 °C. The pellets were either re-suspended in Ni$^{2+}$-affinity buffer A (500 mM NaCl, 50
86 mM Tris-HCl (Melford), 10 % glycerol; pH 8.0) or placed at -20 °C for short-term storage.

2.2.2.2 A. gambiae FMN and A. gambiae FAD expression in LB media

The expression protocol for A. gambiae FMN and FAD binding domains is identical as above (method 2.3.3.1) with the following alteration: The selective media contains the antibiotic kanamycin (32 µg/mL) instead of ampicillin.

2.2.2.3 A. gambiae CPR expression in minimal media

Minimal media solutions A and B were made up separately and 10 mL solution B was added to every 1 L solution A. To make 1 L minimal media solution A required: 12.5 g Na₂HPO₄ (anhydrous), 7.5 g KH₂PO₄ (anhydrous); pH 7.2. This was prepared using Reverse Osmosis (RO) H₂O and autoclaved. To make 10 mL minimal media solution B required: 4 g glucose, 1 g NH₄Cl, 0.24 g MgSO₄.7H₂O, 0.02 g CaCl₂.2H₂O, 0.1 g ampicillin and 0.01 g thiamine-HCl (Duchefa Biochemie). Chloramphenicol (34 µg/mL) was also added. This was also prepared using RO H₂O and passed through a 0.22 µm sterile filter prior to use. Once prepared, and suitably sterilised, solutions A and B
were mixed together and 2 mL inoculated with a single transformed bacterial colony containing the desired expression vector. Over the course of the day, this culture was gradually increased in volume and a 50 mL culture left to grow overnight. This culture was harvested by centrifugation at 8,000 x g for 10 min at 4 °C and the pellet re-suspended in 5 mL fresh minimal media (solutions A and B). Using this re-suspension, 1 L fresh minimal media (solutions A and B), containing 1 mM riboflavin, was inoculated to an OD<sub>600</sub> = 0.05-0.1 and divided equally between 4 x 2 L flasks. These were transferred to a 37 °C incubator and allowed to grow, with shaking at 170 r.p.m, until an OD<sub>600</sub> = 0.7 was achieved. Typically this took approximately 4 hr. Protein expression was induced by addition of 1 mM IPTG and the cultures transferred to a 25 °C incubator, shaking at 170 r.p.m, for 16 hr. The bacterial cells were harvested by centrifugation at 8,000 x g for 10 min at 4 °C. The pellets were either re-suspended in Ni<sup>2+</sup>-affinity buffer A (500 mM NaCl, 50 mM Tris-HCl (Melford), 10 % glycerol; pH 8.0) or placed at -20 °C for short-term storage.
2.2.2.4 A. gambiae FMN and A. gambiae FAD expression in minimal media

The expression protocol for A. gambiae FMN and FAD-binding domains is identical as above (method 2.3.3.3) with the following alteration: Minimal media solution B contained the antibiotic kanamycin (32 mg in 10 mL) instead of ampicillin.

2.2.3 Protein Purification Methods

2.2.3.1 Cell Lysis

The Ni$^{2+}$-affinity buffer A re-suspension (section 2.3.3) was added to with an EDTA-free protease inhibitor tablet and also DNase1 from bovine pancreas (12.5 µg/mL) (Sigma) prior to cell lysis. Cells were lysed by French Press using the Sim Aminco French Pressure Cell at 1000 PSI. The lysate was centrifuged at 20,000 x g for 30 min at 4 °C and the supernatant passed through a 0.22 µm syringe filter prior to purification.
2.2.3.2 Ni\textsuperscript{2+}-affinity chromatography

Ni\textsuperscript{2+}-affinity chromatography was performed using a 5 mL HisTRAP HP column (GE Healthcare) using the AKTA purifier system. Prior to column equilibration, 50 mL MilliQ H\textsubscript{2}O was washed through the column at a flow rate of 2.5 mL/min. Following this, the column was equilibrated using, firstly, 50 mL buffer A (500 mM NaCl, 50 mM Tris-HCl (Melford), 10 % glycerol; pH 8.0) before 25 mL buffer B (500 mM NaCl, 50 mM Tris-HCl (Melford), 1 M imidazole, 10 % glycerol, pH 8.0) was washed through. A further 25 mL buffer A was passed through the column to complete the equilibration. All equilibration washes were at a flow rate of 2.5 mL/min. The filtered supernatant (method 2.4.1) was loaded onto the column at a flow rate of 1 mL/min, the unbound material collected and the column was washed with 25 mL of buffer A. Increasing concentrations of imidazole; 10 mM, 20 mM and 40 mM respectively, were then washed through the column at 2 mL/min (30 mL each) before the bound protein was eluted, again at 2 mL/min, using 250 mM imidazole and the peak fractions analysed.
2.2.3.3 Desalting of samples using a PD-10 column

Desalting and buffer exchange of protein samples were performed using a Sephadex G-25 Medium PD-10 desalting column (GE Healthcare). The column was equilibrated with 25 mL of the desired buffer before sample application. Prior to loading, samples were prepared with an excess of additional flavin (either FMN, FAD or both depending on the sample). This was done before each desalting step during the purification procedure. Between 1 mL and 2.5 mL of sample was loaded to the column and the flow through discarded. To elute the sample, 3.5 mL of the equilibration buffer was applied to the column and the sample collected.

2.2.3.4 Anion exchange chromatography

Final clean-up of samples was performed using a 1 mL MonoQ 5/5 anion exchange column (GE Healthcare) using the AKTA purifier system. Prior to column equilibration, 10 mL MilliQ H₂O was washed through the column at a flow rate of 1 mL/min. Following this, the column was equilibrated using firstly 10 mL anion exchange wash buffer (20 mM Tris-HCl (Melford), 0.2 mM DTT, 1 mM EDTA, 1 mM benzamidine HCl (Sigma); pH 7.5) before 5 mL anion exchange elution buffer (20 mM Tris-HCl (Melford), 0.2 mM DTT, 1 mM EDTA, 1 mM
benzamidine HCl (Sigma), 1M KCl; pH 7.5) was washed through. To finish the equilibration, 5 mL wash buffer was washed through the column, all equilibration steps were performed at 1 mL/min. The sample was injected onto the column and the unbound material was collected. The bound protein was eluted at 1 mL/min with a 0-500 mM KCl gradient over 20 mL and the peak fractions analysed. Cruder protein samples were processed using a 5 mL HiTrap Q FF anion exchange column (GE healthcare). The method used was identical to that for the MonoQ 5/5 column, however, the volumes of buffer required were scaled up 5-fold to compensate for the increased volume of the column. Samples eluted off the HiTrap Q FF column were further processed on the MonoQ 5/5 column if required.

2.2.3.5 Size exclusion chromatography

Size exclusion chromatography was performed using a 320 mL HiLoad 26/60 Superdex 75 column. Prior to use the column was calibrated using molecular weight standards (). The column was initially washed at 1 mL/min with 350 mL MilliQ H₂O followed by equilibration using 350 mL gel filtration buffer (50 mM Tris-HCl (Melford), 50 mM NaCl; pH 7.0). The sample was injected onto the column and run at 1 mL/min. Elution samples were collected after the void volume (110 mL) and peak fractions analysed.
2.2.3.6 His-tag removal using thrombin

Prior to his-tag removal, the protein sample was exchanged into thrombin cleavage buffer (150 mM NaCl, 20 mM Tris-HCl (Melford), 2.5 mM CaCl$_2$; pH 8.4) using a PD-10 desalting column (method 2.2.3.3). Thrombin (Sigma) was added to the protein sample in a ratio of 2000:1 (w/w) protein:thrombin. The thrombin used contained 0.324 µg/NIH unit. This was incubated at room temperature, with slow shaking, for 2 hr. The reaction was stopped by addition of 60 µg/mL PMSF and the sample applied to a Ni$^{2+}$-affinity column to removed free his-tags and uncut protein. The untagged protein did not bind to the Ni$^{2+}$-affinity column and was collected in the flow through.

2.2.3.7 His-tag removal using TEV protease

Prior to his-tag removal, the protein sample was exchanged into cleavage buffer (500 mM NaCl, 50 mM Tris-HCl (Melford), 10 % glycerol; pH 8.0) using a PD-10 desalting column (method 2.2.3.3). TEV protease was added to the protein sample in a ratio of 20:1 (w/w) protein:TEV. This was incubated at room temperature, with slow shaking, overnight to allow cleavage to occur. To remove free his-tags
and uncut protein, the sample was loaded back onto a Ni\(^{2+}\)-affinity column and the flow through was collected.

2.2.3.8 Concentration of protein samples

Protein samples were concentrated using Amicon Ultra Centrifugal Filter Units (Millipore). For larger volumes, units with a capacity of 15 mL were used whilst for smaller volumes, 4 mL units were employed. The different sized units worked and were used in an identical fashion. The protein samples were applied to the units and centrifuged at 4,500 x g. Centrifugation was allowed to occur for 10 min before the remaining sample mixed to prevent excessive sticking of protein to the membrane. This process of centrifugation followed by mixing was used until the sample was concentrated to the desired volume. Throughout the process, the flow through was analysed to ensure no protein of interest passed through the membrane. For full length proteins (A. gambiae CPR, human CPR), units with a 50 kDa cutoff membrane were used. For FAD-binding domains, units with a 30 kDa cutoff membrane were used whilst for FMN-binding domains, units with a 10 kDa cutoff membrane were used. Units were completely reusable providing the membrane was kept moist during storage.
2.2.4 Analytical Methods

2.2.4.1 Bradford protein assay

Protein concentrations were determined using the Bradford Protein Assay (Bradford, 1976). A standard curve was generated using a series of known concentrations (0-2 mg/mL) of BSA. 1 mL Bradford reagent (Sigma) was added to 10 µL protein sample and left to incubate at room temperature for 10 min. The absorbance was read at 595 nm and the protein concentration of unknown samples determined by consultation of the BSA standard curve. All readings were taken using a Cary 300 Bio U.V visible spectrophotometer and the machine zeroed with 10 µL of protein sample buffer was mixed with 1 mL reagent.

2.2.4.2 SDS PAGE

10 mL of resolving gel was made to provide two 0.75 mm 15 % polyacrylamide minigels. The resolving gel comprised 5 mL 30 % acrylamide/bis solution, 2.5 mL distilled H₂O, 2.4 mL 1.5 M Tris; pH 8.8, 100 µL 10 % (w/v) SDS, 75 µL 10 % (w/v) ammonium persulphate, 7.5 µL TEMED (Sigma). Soon after the addition of TEMED, the mixture was gently but thoroughly mixed to avoid bubbles and promptly poured between the assembled plates. Once the resolving gel had been poured, the gel was overlaid with a thin layer of
butan-2-ol and polymerisation allowed to occur for approximately 40 min. Once the gel was fully polymerised, the butan-2-ol was poured off, the gel was rinsed with distilled water and carefully blotted dry. Once dry, a 4 % stacking gel was poured on top of the resolving gel. 10 mL of a 4 % resolving gel comprised 6.1 mL distilled H₂O, 2.4 mL 0.5 M Tris; pH 6.6, 1.3 mL 30 % acrylamide/bis solution, 100 µL 10% (w/v) SDS, 75 µL 10 % (w/v) ammonium persulphate, 7.5 µL TEMED (Sigma). Following gentle mixing, the mixture was poured onto the set resolving gel and a clean comb added to provide the wells for sample loading. The stacking gel was again left to polymerise for approximately 20 min. After polymerisation, the combs were removed and the wells were well rinsed with distilled H₂O. Samples were prepared by addition of 2 x SDS-PAGE sample buffer (50 µM Tris; pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 4 % (v/v) β-mercaptoethanol, 0.1 % (w/v) bromophenol blue, H₂O) and denatured at 95 °C for 5 min. Prior to loading, the samples were centrifuged at 14,000 x g for 2 min. The cast gels were placed in the Protean II gel running kit (BioRad) and placed in a tank containing gel running buffer (2.5 mM Tris-Base, 25 mM Glycine, 0.01 % SDS.). The central cavity of the apparatus was filled with gel running buffer and 7.5 µL of the sample loaded into the wells using a syringe (Hamilton) along with 5 µL of low range protein standards (Sigma). Samples were allowed to run at 200 V until the
bromophenol blue dye reached the bottom of the gel; typically this took 45-50 min.

2.2.4.3 Coomassie Brilliant Blue staining

As soon as SDS-PAGE had been performed, the gels were placed in a solution of Coomassie G250 (0.1 % Coomassie G250, 45 % methanol, 10 % acetic acid, distilled H₂O). Incubation was at room temperature, with slow shaking, for 1 hr. The Coomassie G250 solution was removed and the gels washed with distilled H₂O before being placed in distilled H₂O containing methanol and returned to the shaker. The solution was changed regularly until complete destaining of the background had occurred.

2.2.4.4 Gel imaging

Gel images were acquired using a GS-710 Calibrated Imaging Densitometer (BioRad).
2.2.4.5 Electrospay mass spectrometry

For electrospay mass spectrometry, aliquots of purified protein samples were taken to Manchester Interdisciplinary Biocentre for analysis.
Chapter 3

3. CLONING, EXPRESSION, PURIFICATION AND ENZYME CHARACTERISATION

3.1 Introduction

Over the last 40 years, there has been considerable volume of biochemical and biophysical experimentation on cytochrome P450 reductases from a number of different species. The most widely studied CPR’s are those from human, house fly and rat; the latter providing the first crystal structure, albeit of the N-terminal truncated enzyme (Wang et al., 1997). Little is known about the CPR’s from mosquito species and it is only recently that work has been carried out on Anopheline reductases such as A. minimus (Sarapusit et al., 2008). To date the biochemical and biophysical properties of A. gambiae CPR have yet to be reported.
With the aim of gaining biophysical information about *A. gambiae* CPR, a number of factors need to be considered. The work presented here is the first example of a CPR and its individual FMN-binding and FAD-binding domains being expressed and purified with a view to biophysical analysis. In order to fully understand the requirements of generating such biophysical information, it is important to appreciate the biochemical behaviour of the enzyme. With this in mind, the soluble, full length CPR as well as the isolated FMN and FAD-binding domains have been purified and expressed to ascertain primary biochemical behaviour and to serve as a forerunner to more detailed structural analysis.

To achieve this, recombinant *A. gambiae* CPR, FMN and FAD-binding domains have been expressed in a bacterial expression system and purified using affinity and anion exchange chromatographies. Stable isotope labelling techniques to incorporate $^2$H, $^{15}$N and $^{13}$C for NMR experiments have also been employed for each protein. The spectral characteristics and levels of flavin incorporation in human CPR are widely known and this enzyme has been brought into the study as a comparative aid.
3.2 Methodologies and Results

3.2.1 Expression and Purification

To perform biochemical and biophysical analysis on A. gambiae CPR and its FMN and FAD-binding domains, first an efficient method for recombinant protein production must be developed. The expression system used for these expressions was bacterial utilising competent E. coli Codon Plus cells (Stratagene) to express the plasmid components. Transformed bacteria were cultured at 37 °C before protein expression, induced by addition of 1 mM IPTG, overnight at 30 °C. Harvested bacteria were lysed by French Press and samples centrifuged for at 20,000 x g for 30 min at 4 °C. Following cell lysis, it was imperative that DNase was added to remove problematic DNA, particularly when the sample was for biophysical analysis.

The purification protocol was modelled on that for the human FMN and FAD-binding domains used by Zhao et al., 1996. It is a two step purification protocol employing Ni²⁺- affinity followed by anion exchange chromatography columns. This protocol was used as it was successful and reliable in producing samples for NMR spectroscopy which is a major requirement for sample production in this work.
3.2.1.1 Purification of full length CPR

For the purposes of this study, an N-terminal truncated *A. gambiae* was utilised. The recombinant protein was derived from a pET15b expression vector and supplied by Dr. Mark Paine (Liverpool School of Tropical Medicine). This enzyme lacked the first 63 amino acid membrane anchor region, meaning that the enzyme was readily expressed and found in the soluble supernatant following bacterial cell lysis. The first purification step of the soluble material involved a Ni$^{2+}$ affinity column and the chromatogram shown in figure 3.1. The major peak is observed during the 250 mM imidazole elution step with small peaks detected at 280 nm following each of the 10, 20 and 40 mM imidazole wash steps. Since the flavin cofactors also absorb light at 380 and 450 nm, these wavelengths were also analysed. At these wavelengths, the major elution peak was still clearly visible but the smaller peaks detected in the wash steps were not. This suggests that very little, if any *A. gambiae* CPR was lost during column washing (Figure 3.1). Minimal losses were reported during the desalting step or following thrombin cleavage of the N-terminal His-tag. Following reverse purification on the Ni$^{2+}$ affinity column, anion exchange chromatography (AEX) was performed. Figure 3.2 shows the chromatogram following AEX with a major peak detected after 330
**Figure 3.1. Chromatograms of *A. gambiae* CPR following Ni\textsuperscript{2+}-affinity purification.** Sample was applied to a 5 mL His-Trap Ni\textsuperscript{2+}-affinity column in 500 mM NaCl, 50 mM Tris-HCl, 10 % glycerol; pH 8.0. Bound protein was eluted using the same buffer containing 250 mM imidazole. The traces are from the same sample detected at A = 280 nm; B = 380 nm and C = 450 nm.
Figure 3.2. Chromatogram of *A. gambiae* CPR following anion exchange purification and SDS-PAGE of purified sample. **A** = Anion exchange chromatogram. Sample was applied to a 1 mL MonoQ 5/5 column in 20 mM Tris-HCl 0.2 mM DTT, 1 mM EDTA, 1 mM benzamidine HCl; pH 7.5. Bound protein was eluted using the same buffer containing KCl over a 0-500 mM gradient and detected at 280 nm. **B** = SDS-PAGE analysis of purified peak sample.
mM KCl had passed through. This detection was confirmed by the 380 and 450 nm curves (Figure 3.2) confirming presence of flavin. Purified enzyme was bright yellow in colour, indicating flavin cofactor incorporation, and the purity was checked by SDS-PAGE. The fully purified sample contained a single band of approximately 70 kDa, the expected molecular weight for N-terminal truncated *A. gambiae* CPR (Figure 3.2). Protein concentration was calculated by Bradford Assay and the standard yield was approximately 20 mg purified enzyme per litre of bacterial culture. The sample was verified as *A. gambiae* CPR by mass spectrometry (Appendix A)

3.2.1.2 Dissection of *A. gambiae* CPR into FMN and FAD-binding domains

The N-terminal his-tagged pET15b *A. gambiae* CPR expression vector was used as template DNA for dissection into the constituent FMN and FAD-binding domains. Primers were designed to the domains based on the rat CPR crystal structure and sequence alignments to isolate the positions of the domain boundaries. Initially, primers were designed and constructs incorporating the domain boundaries, with no additional residues at either terminus, were generated. One obstacle to overcome was that since the restriction enzymes of choice for pET15b were Nde1
and BamH1, a silent A to C mutation had to be incorporated into the residue I617 to remove a CATATG Nde1 binding sequence from within the FAD-binding domain. Taking into account the melting points of the primers, the PCR protocol was: Step 1 – 1 cycle of a 95°C initial step for 2 min; Step 2 – 30 cycles of a 15 s 98 °C denaturation, a 30 s 45 °C annealing and a 15 s 72 °C elongation; Step 3 – a 72 °C final extension for 1 min; Step 4 – Hold step at 4 °C.

The agarose gel of the PCR products showed bands at the correct size; approximately 550 bp for FMN and 1300 bp for FAD, suggesting that cloning had been successful. Plasmid sequencing of the FAD-binding domain vector failed to show the full correct insert with errors in all bases after the forced mutation: this rendered the plasmid unsuitable for use. The sequencing on the FMN-binding domain plasmid was successful.

The FMN-binding domain was expressed in *E. coli* and purified by Ni²⁺-affinity and anion exchange chromatographies. Following purification however, the FMN-binding domain appeared as a double banded species which was present throughout the process. SDS-PAGE analysis shows the presence of two bands with the second band approximately 1-2 kDa below the first. The same two bands are seen even after His-tag removal and the molecular weight difference
Figure 3.3. Purified *A. gambiae* FMN-binding domain, pre- and post- his-tag cleavage. Lane 1 = Molecular weight markers; Lane 2 = *A. gambiae* FMN-binding domain pre-his-tag removal; Lane 3 = *A. gambiae* FMN-binding domain post-his-tag removal. The molecular weight protein ladder is shown in kDa.
between the two bands was maintained. Unsuccessful attempts were made to separate the bands by anion exchange chromatography techniques and since the molecular weight difference was deemed too small to separate by size exclusion chromatography, the expression vectors were redesigned.

Due to the problems encountered with both the FMN and FAD-binding domains, an alternative approach was employed. The main alteration in this approach was to change the expression vector from a pET15b to a pETM-11 vector. The reason for this was to eliminate the use of NdeI restriction enzyme and thus eliminate the problem of incorporating a mutation within this domain and to move away from thrombin in the removal of the His-tag since pETM-11 contains a site for the much more specific TEV protease. Furthermore, in an attempt to solve the problem of the double banded FMN-domain, extended FMN and FAD-binding domain constructs were made. These incorporated a small section of the linker domain in each case as a possible attempt to stabilise the purified domain. The design of these constructs is described (Figure 3.4) and the primers used to generate them are also described (Appendix B). Due to the similarity in melting point of all the manufactured primers, the same PCR protocol could be used throughout the process and involved; Step 1 – 1 cycle of a 95 °C initial step for 2 min; Step 2 – 30 cycles of a 15 s 95 °C denaturation, a 15 s
55 °C annealing and a 40 s 72 °C elongation; Step 3 – a 72 °C final extension for 10 min; Step 4 – Hold step at 4 °C.

The agarose gel of the PCR products, as before, showed bands at the correct sizes meaning cloning was successful. The bands seen in Figure 3.5 are for the extended FMN and FAD-binding domain in each case; approximately 600 bp for FMN and 1300 bp for FAD-binding domain. Following successful ligation into the pETM-11 vector, the constructs were sequenced which confirmed correct cloning before then being taken forward for protein expression and purification (Figure 3.5).
Figure 3.4. A schematic representation of the generated FMN-binding and FAD-binding domain constructs of *A. gambiae* CPR. A, B and C are the FMN-binding domain constructs where A is the entire domain incorporating a section of the inter-domain linker region, B is simply the entire domain and C is a slightly shortened domain. D and E are the FAD-binding domain constructs both of which are the entire domain incorporating a section of the inter-domain linker region with E including a slightly longer section that D.
Figure 3.5. Agarose gel analysis of *A. gambiae* FMN-binding domain and FAD-binding domain PCR products. Lane 1 = DNA ladder, with sizes shown in bp; Lane 2 = FMN-binding domain PCR product; Lane 3 = FAD-binding domain PCR product; Lane 4 = Negative control (without template DNA).
3.2.1.2.1 Purification of extended FMN binding domain

The pETM-11 based FMN-binding domain expression vector was used to transform competent *E. coli*. The purification protocol was identical to that used for the full length soluble CPR involving a Ni\(^{2+}\)-affinity chromatography step followed by an anion exchange chromatography step. The chromatogram for the FMN-binding domain following Ni\(^{2+}\) affinity chromatography is shown, again analysed at the wavelengths 280, 380 and 450 nm. As with the full length CPR, the major peak was observed during the 250 mM imidazole elution step (Figure 3.6). The same peak is observed when detected at 380 and 450 nm and these traces show little by way of lost protein during the low imidazole concentration wash steps (Figure 3.6). Minimal losses were observed following desalting, His-tag cleavage and reverse purification on the Ni\(^{2+}\)-affinity column.

Anion exchange chromatography yielded a major peak during the KCl elution gradient. The purified sample was eluted from the column at 380 mM KCl. The purity of the sample was analysed by SDS-PAGE. Unlike with the shorter FMN-binding domain, the elongated domain was purified as a single band at the expected molecular weight of approximately 21 kDa (Figure 3.7). Protein concentration was determined using the Bradford Assay and a typical yield for the FMN-binding domain was in the region of 12-15 mg purified enzyme per
litre of bacterial culture. The molecular weight of the FMN-binding domain was calculated by mass spectrometry to be 20,755 Da (Appendix C). This value is a lot lower than the calculated 22,631 Da for the domain which could be attributed to degradation of the sample which was sent for analysis due to its age.
Figure 3.6 Chromatograms of *A. gambiae* FMN-binding domain following Ni$^{2+}$-affinity purification. Sample was applied to a 5 mL His-Trap Ni$^{2+}$-affinity column in 500 mM NaCl, 50 mM Tris-HCl, 10% glycerol; pH 8.0. Bound protein was eluted using the same buffer containing 250 mM imidazole. The traces are from the same sample detected at A = 280 nm; B = 380 nm and C = 450 nm.
Figure 3.7. Chromatogram of *A. gambiae* FMN-binding domain following anion exchange purification and SDS-PAGE of purified sample. **A** = Anion exchange chromatogram. Sample was applied to a 1 mL MonoQ 5/5 column in 20 mM Tris-HCl 0.2 mM DTT, 1 mM EDTA, 1 mM benzamidine HCl; pH 7.5. Bound protein was eluted using the same buffer containing KCl over a 0-500 mM gradient and detected at 280 nm. **B** = SDS-PAGE analysis of purified peak sample.
3.2.1.2.2 Purification of extended FAD-binding domain

The expression and purification of the *A. gambiae* FAD-binding domain was the same as for the full length and FMN-binding domains; a two-step purification including Ni\(^{2+}\)-affinity and anion exchange chromatographies. The chromatogram following Ni\(^{2+}\)-affinity chromatography shows a major peak after the 250 mM imidazole elution step (Figure 3.8). The difference between the protocol for the FAD-binding domain and that for the FMN-binding domain and full length CPR was in the wash steps. The FAD-binding domain was shown to bind less strongly to the Ni\(^{2+}\) affinity column under these conditions. Because of this, the wash steps included just 10 and 20 mM imidazole respectively with the 40 mM imidazole wash step removed.

The FAD-binding domain was a lot more problematic than the other *A. gambiae* CPR derived samples. The protein showed a tendency to precipitate throughout the purification process, particularly during the desalting steps and following TEV-protease cleavage. During desalting, despite yellow protein being eluted from the PD-10 column, there were moderate levels of precipitation making the sample look cloudy. However, once centrifuged, the pelleted precipitate was distinctly clear rather than yellow in colour. The supernatant was re-applied to the PD-10 column and eluted as a clear, yellow sample.
Once the sample was satisfactorily stable it was applied to the anion exchange column.

The chromatogram following anion exchange chromatogram showed a major peak during the KCl elution gradient. The sample was shown to elute at around 80 mM KCl, a lot earlier than is observed in the FMN-binding domain and full length CPR which eluted at over 300 mM KCl. The peak was assessed by SDS-PAGE for purity and a single band at the expected molecular weight of around 50 kDa was seen (Figure 3.9). Protein concentration was assessed by Bradford Assay and a typical yield was approximately 10-15 mg purified enzyme per litre of bacterial culture. The FAD-binding domain was calculated by mass spectrometry to be 48,628 Da. (Appendix C). This value is close to the predicted value for this domain of 48,712 Da and the observed difference could be attributed to potential cysteine or methionine modifications such as carbamidations, oxidations or carboxylations.
Figure 3.8. Chromatograms of *A. gambiae* FAD-binding domain following Ni\(^{2+}\)-affinity purification. Sample was applied to a 5 mL His-Trap Ni\(^{2+}\)-affinity column in 500 mM NaCl, 50 mM Tris-HCl, 10 % glycerol; pH 8.0. Bound protein was eluted using the same buffer containing 250 mM imidazole and detected at 280 nm.
Figure 3.9. Chromatogram of *A. gambiae* FAD-binding domain following anion exchange purification and SDS-PAGE of purified sample. A = Anion exchange chromatogram. Sample was applied to a 5 mL HiTrap QFF column in 20 mM Tris-HCl 0.2 mM DTT, 1 mM EDTA, 1 mM benzamidine HCl; pH 7.5. Bound protein was eluted using the same buffer containing KCl over a 0-500 mM gradient and detected at 280 nm. B = SDS-PAGE analysis of purified peak sample.
3.2.1.3 Purification of human CPR and FMN-binding domain

The expression and purification of the human full length CPR and isolated FMN-binding domain was identical to that for the *A. gambiae* equivalents. Ni$^{2+}$- affinity chromatography of both the full length CPR and the FMN-binding domain yielded a major peak in the 250 mM imidazole elution step (Figure 3.10) with minimal losses reported during the lower concentration imidazole wash steps in each case. There was a major peak following anion exchange chromatography in each case (Figure 3.11) with respective elutions after approximately 325 mM KCl for human CPR and 380 mM KCl for human FMN-binding domain. These values were very similar, almost identical to those observed for the *A. gambiae* CPR suggesting that with respect to this purification strategy, the mosquito and human sample behave in a similar fashion.
Figure 3.10. Chromatograms of human CPR and isolated FMN binding domain following Ni\textsuperscript{2+}-affinity purification. A = human CPR; B = human isolated FMN-binding domain. Samples were applied to a 5 mL His-Trap Ni\textsuperscript{2+}-affinity column in 500 mM NaCl, 50 mM Tris-HCl, 10 % glycerol; pH 8.0. Bound protein was eluted using the same buffer containing 250 mM imidazole and detected at 280 nm.
Figure 3.11 Chromatogram of human CPR and isolated-FMN-binding domain following anion exchange purification and associated SDS-PAGE analysis. A = Human CPR, inset = SDS-PAGE of purified sample; B = Human FMN-binding domain, inset = SDS-PAGE of purified sample. Samples were applied to a 1 mL MonoQ 5/5 column in 20 mM Tris-HCl 0.2 mM DTT, 1 mM EDTA, 1 mM benzamidine HCl; pH 7.5. Bound protein was eluted using the same buffer containing KCl over a 0-500 mM gradient and detected at 280 nm. For the SDS-PAGE, the molecular weights of the markers are shown in kDa. The peak in panel B (FMN-binding domain) appears truncated due to column overloading leading to over an above maximal U.V detection being achieved.
3.2.2 Spectral properties

When FMN and FAD flavins are bound to cytochrome P450 reductases, they cause unique spectral properties to be observed in these enzymes under aerobic conditions. The FMN and FAD cofactors absorb light at around 380 and 450 nm giving rise to characteristic spectra. The *A. gambiae* FMN-binding domain was scanned between 700-300 nm resulting in such a spectral pattern (Figure 3.12 A). An almost identical spectra was achieved when the human FMN-binding domain was scanned under the same aerobic conditions (Figure 3.12 B). These spectra confirm presence of the flavin bound holoenzyme.

The propensity of CPRs to adopt an ‘air-stable’ semiquinone state is well established and this is also the case in *A. gambiae* CPR. Under aerobic conditions, full length *A. gambiae* CPR shows the characteristic spectra with absorption peaks at around 380 and 450 nm (Figure 3.13). Immediately following the addition of a stoichiometric amount of NADPH, there is an immediate change in the spectral properties. The 450 nm, and to a lesser extent the 380 nm, peaks decrease in absorbance and there is a distinctive rise in the absorbance in the 500-700 nm region (Figure 3.13 B). The same sample, scanned after 30 min, still showed an in absorbance in the 500-700 nm region.
Figure 3.12. The absorption spectra of the FMN-binding domain from *A. gambiae* and human CPR’s. A = *A. gambiae* FMN-binding domain; B = Human FMN-binding domain. Samples were scanned under aerobic conditions using a Cary400 UV spectrophotometer between 300 and 700 nm wavelengths.
Figure 3.13. The formation of the ‘air-stable’ semiquinone in *A. gambiae* CPR. A (blue) = oxidised *A. gambiae* CPR; B (red) = immediately following addition of NADPH; C (green) = 30 min following addition of NADPH. Samples were scanned under aerobic conditions using a Cary400 UV spectrophotometer between 300 and 700 nm wavelengths.
with respect to spectrum A and only partial restoration of the 380 and 450 nm absorptions (Figure 3.13). Spectrum C remained the same even when the sample was left overnight confirming that *A. gambiae* does indeed adopt an ‘air-stable’ semiquinone state in aerobic conditions.

3.2.3 Analysis of flavin content – comparing the human and the *A. gambiae* CPR

FMN and FAD cofactor interaction with CPR is necessary for correct function. Residues in and around the flavin binding regions are highly conserved across many species and mutations in these key residues can lead to impaired flavin incorporation and catalytic function. In most cases, the ratio of each flavin to protein is 1:1 in CPR (Vermilion and Coon, 1978) but there are exceptions, most notably in the *A. minimus* mosquito where the ratio is 0.63:1 for FAD and as low as 0.51:1 for FMN (Sarapusit *et al.*, 2008). It is important to assess the level of flavin incorporation in CPR samples. Studying a sample which is fully saturated with flavin cofactor is of particular interest from a biophysical perspective as it removes any potential risks of protein instability or slight shifts in conformation which may arise in flavin deficient samples.
Flavin cofactors (FMN and FAD) were isolated from the *A. gambiae* CPR by precipitation of the protein. 100 µL *A. gambiae* CPR was prepared to a concentration of 0.1 mg/mL and the sample incubated at 95 °C for 5 min. Precipitated samples were centrifuged at room temperature at 20,000 x g for 5 min and the supernatent taken for HPLC analysis. Buffer samples to be analysed were treated in exactly the same way as the protein samples. Isolated flavin samples transferred to HPLC vials. A range of FMN and FAD standards were prepared in the same buffer conditions as the protein sample. 10 µL sample (or standards) were loaded into a mobile phase of 50 mM ammonium acetate; pH 4.5 with 20 % (v/v) acetonitrile for separation on a 250 mm C\textsubscript{18} column (Acclaim®120, Dionex) at 23 °C. The flow-through was analysed by absorption spectroscopy and fluorescence (excitation 450 nm, emission 525 nm) for 7 min before loading the next sample. Samples were diluted 10-fold prior to loading onto the column. Quantification was determined by integration of the fluorescence peaks and compared to 250 nM and 50 nM FMN and FAD standard sample peaks.

The HPLC method used shows good separation of the FMN and FAD with FAD eluting at around 3 min compared to FMN eluting at 4 min. In comparing the peak areas alone, it is clear that the human CPR has a
Figure 3.14. HPLC analysis of the \textit{A. gambiae} and human flavin content. \textbf{A} = comparison of \textit{A. gambiae} CPR and human CPR flavin content. \textit{Blue trace:} 250 nM flavin standard, \textit{Red trace:} Boiled \textit{A. gambiae} CPR, \textit{Green trace:} Boiled human CPR \textbf{B} = comparison of \textit{A. gambiae} CPR flavin contents from different preparations. \textit{Blue trace:} 500 nM flavin standard, \textit{Green trace:} \textit{A. gambiae} CPR purified with no additional flavin, \textit{Red trace:} \textit{A. gambiae} CPR prepared with flavin added during purification. HPLC was carried out on a 250 mm C\textsubscript{18} column (Acclaim®120, Dionex) at 23 °C using 50 mM ammonium acetate; pH 4.5 with 20 % (v/v) acetonitrile as the mobile phase. Fluorescent emission was measured at 525 nm.
Table 3.1. Flavin concentration of *A. gambiae* and human CPR preparations. *A. gambiae* \(^a\) and human samples were prepared with both riboflavin in the bacterial media and exogenous flavin during purification; *A. gambiae* \(^b\) sample was prepared with riboflavin in the bacterial expression media but no additional exogenous flavins. *A. gambiae* \(^c\) sample was prepared with neither riboflavin in the bacterial expression media nor additional exogenous flavin.

<table>
<thead>
<tr>
<th>CPR sample</th>
<th>Protein Concentration (nmol/mL)</th>
<th>(a = \text{FAD concentration (nmol/mL)}) (b = \text{mol. FAD/mol. protein})</th>
<th>(a = \text{FMN concentration (nmol/mL)}) (b = \text{mol. FMN/mol. protein})</th>
<th>Total flavin:protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gambiae</em> (^a)</td>
<td>23.5</td>
<td>(a = 18.8 \pm 0.2) (b = 0.80 \pm 0.01)</td>
<td>(a = 16.9 \pm 0.2) (b = 0.72 \pm 0.01)</td>
<td>1.52:1</td>
</tr>
<tr>
<td>Human</td>
<td>38.4</td>
<td>(a = 35.2 \pm 0.7) (b = 0.92 \pm 0.02)</td>
<td>(a = 33.9 \pm 1.2) (b = 0.88 \pm 0.03)</td>
<td>1.80:1</td>
</tr>
<tr>
<td><em>A. gambiae</em> (^b)</td>
<td>16.2</td>
<td>(a = 11.4 \pm 0.2) (b = 0.70 \pm 0.01)</td>
<td>(a = 9.0 \pm 0.1) (b = 0.56 \pm 0.01)</td>
<td>1.26:1</td>
</tr>
<tr>
<td><em>A. gambiae</em> (^c)</td>
<td>15.6</td>
<td>(a = 6.5 \pm 0.3) (b = 0.42 \pm 0.02)</td>
<td>(a = 3.4 \pm 0.1) (b = 0.22 \pm 0.01)</td>
<td>0.86:1</td>
</tr>
</tbody>
</table>
greater content of both FMN and FAD in comparison to *A. gambiae* CPR (Figure 3.14 Panel A). For full quantification, however, the flavin content in relation to the original protein samples was calculated.

The peak areas of the sample FMN and FAD peaks were directly compared to the flavin standard peaks to give a concentration of flavin loaded onto the column. This was then used and by taking into account sample dilution the concentration of the original sample could be calculated. For ease of direct comparison, the reported units for flavin and protein are identical. For example, *A. gambiae* in table 3.1, the starting concentration of the protein sample was 1.65 mg/mL as calculated by Bradford assay. For a protein of approximately 70 kDa, 1 mg/mL is equivalent to 14.3 μM which in turn is equivalent to 14.3 nmol/mL. Thus 1.65 mg/mL is equal to 23.5 nmol/mL. In this example, the concentration of eluted FAD was 114 nM. Since the sample was diluted 10-fold prior to loading onto the HPLC column, the concentration of the 100 µL prepared sample was therefore 1140 nM. This was derived from 0.1 mg/mL CPR thus in the original 1.65 mg/mL sample there was 18810 nM (18.810 μM). This equates to 18.8 nmol/mL thus from 23.5 nmol/mL CPR there is 18.8 nmol/mL FAD cofactor which is a 0.8:1 ratio. The same calculation was applied to the other samples to generate Table 3.1.
These data confirm that human CPR has a higher concentration of both FMN and FAD than the mosquito enzyme. Having FAD and FMN contents of 0.92:1 and 0.88:1 respectively, the human CPR is almost fully saturated with flavin: the same is not true for A. gambiae CPR. The mosquito enzyme is both FAD and FMN deficient but whilst the FAD content is still reasonably high at 0.80:1, the FMN deficiency is more noticeable. A 0.72:1 FMN:protein ratio in A. gambiae CPR represents an almost 20% decrease in FMN content when compared to human CPR (Table 3.1).

Analysis of the flavin content in CPR has allowed the effect of adding exogenous FMN and FAD during the purification process to be studied. If, between each chromatography step, an excess of FMN and FAD was added to the sample, the flavin contents in A. gambiae CPR were as observed above at 0.80:1 for FAD and 0.72:1 for FMN. If however, the purification process carried out without the addition of exogenous flavin at any stage, the cofactor content is decreased further (Figure 3.14 Panel B). The FAD content falls to 0.70:1 and the FMN drops as low as 0.56:1 (Table 3.1). This suggests that FAD and to a greater extent FMN can be lost during purification and require replenishing throughout. With this in mind, to maximise cofactor content, all samples were prepared with exogenous flavin added during purification.
The addition of riboflavin into the bacterial growth and expression media had a dramatic effect on the flavin levels in *A. gambiae* CPR. Riboflavin is an essential precursor for FMN and FAD production in *E. coli* thus CPR produced in a riboflavin free expression medium is severely depleted in flavin cofactor. Such samples had an FAD content of 0.41:1 whilst the FMN content was 0.22:1; around 70% lower than what is observed in the sample produced with riboflavin and saturated with exogenous flavin during purification (Table 3.1).

This highlights the importance of both the incorporation of riboflavin into the bacterial expression media and the replenishment of lost flavins during purification. Despite having these strategies in place, *A. gambiae* CPR still appears to be flavin depleted, a trait this protein has in common with CPR from the related *A. minimus* (Sarapusit et al., 2008). The fact that these two related mosquito enzymes show this similarity could be a consequence of specialised activities of CPRs in these insects and in order to fulfil these roles may have sacrificed fully effective flavin binding. The reasons for poor flavin incorporation will most likely be elucidated through high-resolution structural determination.
3.2.4 NMR analysis

3.2.4.1 Introduction

Nuclear magnetic resonance spectroscopy (NMR) is a biophysical technique commonly used in the analysis and determination of protein structure. The technique exploits the magnetic spin of certain nuclei; in biological studies, the three most commonly nuclei used in NMR are $^1$H, $^{13}$C and $^{15}$N. All three nuclei have spin number if $\frac{1}{2}$ which means that the nucleus can align either parallel or perpendicular to the magnetic field. The natural abundances of $^1$H, $^{13}$C and $^{15}$N are, respectively, 100%, 1% and 0.01%. To maximise the sensitivity for NMR studies, biological samples are isotopically labelled with $^{15}$N and $^{13}$C. Maximal labelling is required for most experiments. In addition to improving sensitivity, the use of isotopically labelled samples allowed more complicated NMR experiments to be performed; the most beneficial effects of this development is that multi-dimensional NMR experiments can be performed, affording improved resolution of the NMR data.

Another common stable isotope employed in labelling techniques is $^2$H. Replacing protons with deuterons in a protein sample lengthened the transverse relaxation time, $T_2$, of the remaining protons; in other words, the linewidths of the NMR resonance, which has a inverse relationship to $T_2$, are much reduced, thereby giving improved
sensitivity. Deuteration is most pertinent and beneficial for proteins of molecular mass greater than 30kDa.

In the following sections, a description on the application of NMR is given, focussing on issues that are directly relevant to this project rather than providing a comprehensive survey of the various uses of biological NMR.

3.2.4.2 Applications of NMR for Biological Studies

A one-dimensional (1D) proton NMR spectroscopy is a relatively simple experiment in part due to the fact that no isotope labelling is required for analysis. A 1D proton NMR spectrum is a quick and fairly straightforward way of assessing whether a protein is correctly folded or not. This is an important fact as for longer and more complicated experiments, it is vital that the protein sample has been properly prepared and behaves well in the selected sample buffer for prolonged periods in the magnet. Once correct folding under these conditions has been ascertained, more complex and informative experiments can be run.

$^{15}$N labelled samples are prepared by using $^{15}$NH$_4$Cl as the sole nitrogen source in the growth media of E.coli expressing the desired
protein. $^{15}$N labelled sample spectra can be used, as with proton NMR spectra, to further assess the folding of the sample but also to make a judgement on the quality of generated spectra to see whether it is worth expending more time and money preparing more samples for further, potentially more expensive experiments. For example, partial folding and aggregation is better assessed using 2D $^1$H-$^{15}$N HSQC spectra. For relatively small proteins, a $^{15}$N-labelled sample can be sufficient to assign a sample’s backbone resonances. $^{15}$N-labelled samples can also be used in titration experiments with ligands or other proteins as a way of assessing binding sites or conformational shifts during complex formation.

$^{15}$N, $^{13}$C-labelled samples are more expensive to produce as $^{13}$C-labelled glucose is used in bacterial growth media. Triple resonance spectra are used in order to assign both backbone and side chain resonances, provided the spectra are of sufficient quality. In order to determine the structure of a protein, a very high proportion of backbone and side chain assignments are required. Replacing $^1$H with $^2$H in studied samples has the most dramatic effect of all isotope labelling by allowing much larger molecular weight samples to be studied. The incorporation of $^2$H removes the problems of peak overcrowding and overlapping which is apparent in large protein. As the proton magnetisation in a large protein relaxes a lot faster, that is,
there is less time available to detect the signal which in turn broadens the peaks until no signal is detected. Deuteration of protein samples overcomes this problem by lengthening the transverse relaxation, hence, allowing improving the sensitivity of the remaining proton resonances.

3.2.4.3 Structural Studies of A. gambiae CPR

The human CPR FMN-binding domain has been studied by NMR and its resonances assigned. (Barsukov et al., 1997) Much of the structural study on CPRs has been performed using the X-Ray Crystallography method and due to the sheer size of the protein (~70 kDa.) NMR is less widely used for these structural studies. The work described in this thesis is the first time that the A. gambiae CPR has been characterised in some detail using biophysical techniques. It is also the first instance that NMR has been used on a full length CPR as well as its constituent FMN and FAD-binding domains. To fully solve a structure of such a large protein, with the added difficulty of bound flavin cofactors, was unrealistic in the timeframe of this project; however, the work described will serve as important groundwork for future structural analysis of A. gambiae CPR and potentially other related proteins.
3.2.4.3.1 NMR methods

NMR spectra were recorded at 298K on Bruker Avance 600 and Avance 800 MHz spectrometers equipped with $^1$H, $^{13}$C, $^{15}$N triple resonance cryoprobes. The spectra were processed using TopSpin (Bruker) and analysed using CCPN Analysis (Vranken et al., 2005). The sequence specific H$_N$, $^{15}$N, C$_a$, C$_\beta$ and CO backbone assignments were based on the following experiments: 2D $[^{15}$N,$^1$H]-HSQC, 2D $[^{15}$N,$^1$H]-TROSY, 3D HNCA, 3D HNCO, 3D HNCACB, 3D CBCA(CO)NH, 3D HNCOCA and 3D HNCACO Tetramethyl silane (TMS) was used as a chemical shift reference for $^1$H, and $^{15}$N and $^{13}$C shifts were referenced indirectly using the absolute frequency ratios.

3.2.4.3.2 FMN-binding domain

The FMN-binding domain, at approximately 21 kDa, is ideally suited to study by NMR: this has been shown previously with the human equivalent (Barsukov et al., 1997). The fact that the expressed A. gambiae domain could be produced with high yields was also an advantage particularly when isotope labelling was employed.

1D proton NMR was performed on purified samples, primarily to assess whether the expressed protein was correctly folded and behaved sufficiently in the sample buffer. The buffer used for these experiments
was 40 mM phosphate, 0.2 mM DTT; pH 6.8 and NMR data collected at 298 K. The spectrum for the FMN-binding domain confirmed that the sample showed correct and proper folding. This was an important experiment to perform as it meant that further, more expensive samples could be prepared safe in the knowledge that the domain could be produced in a folded state.

Isotope labelling, with both $^{15}$N and $^{13}$C, was successful and subsequent samples showed high levels of labelling without affecting the yield of expressed protein. A $^{15}$N, $^{13}$C double labelled sample was expressed and spectra produced. The spectra were clear with separate clean peaks implying a good quality sample.

However, the best quality spectra were obtained when exogenous FMN was added in order to saturate the protein with FMN and ensure full complexation with the cofactor. The improvement on the quality of the spectrum, with well resolved resonances reminiscent of a properly folded protein implies that the isolated FMN-binding domain suffered, as with full length CPR, from a deficiency in FMN cofactors. The very fact that adding FMN helped with the quality of samples indeed shows that poor flavin incorporation could indeed affect structural integrity of the FMN-binding domain in A. gambiae CPR. Figure 3.15 shows a $^1$H, $^{15}$N HSQC spectrum of the FMN-binding domain following the addition of exogenous FMN to the sample.
Figure 3.15. HSQC spectrum of $^1$H $^{15}$N, -labelled *A. gambiae* isolated FMN-binding domain. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8-298 K.
The addition of FMN yields a good quality spectrum with good levels of peak detection and separation.

Having established that exogenous FMN was required, the next step was to ensure that the protein sample was stable for extended period of up to three weeks in order for high quality NMR data to be collected for structure determination. It was found that over this period and at 25 °C (the optimal compromise temperature between resonance line-widths and protein stability) the protein showed signs of unfolding despite the fact that excess FMN was present in the sample, as assessed by the large free FMN resonances in the spectra. Despite these stability limitations, a complete set of triple resonance data was acquired. Figures 3.16 and 3.17 are CBCA(CO)NH and HNCA projections of the triple resonance data. These data confirm that there is sufficient sensitivity in these samples to obtain complete backbone resonance assignments. Due to time limitations, these assignments were not undertaken as part of the thesis.
Figure 3.16. 3D CBCA(CO)NH HSQC spectrum of \textit{A. gambiae} isolated FMN-binding domain. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8-298 K.
Figure 3.17. 3D HNCA HSQC spectrum of *A. gambiae* isolated FMN-binding domain. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8-298 K.
3.2.4.3 FAD-binding domain

The inherent lack of stability of *A. gambiae* FAD-binding domain was a clear potential obstacle in performing biophysical analysis. The sample buffer used for experiments was the same as for the FMN-binding domain and despite the stability issues during purification, samples for NMR were produced. A 1D proton NMR spectra confirmed that the expressed protein was correctly folded (Figure 3.18) and labelled samples were hen produced.

The FAD-binding domain is rather large at just under 50 kDa thus simply assessing protonated samples would likely lead to poor quality spectra. With this in mind, in an attempt to improve sensitivity, a deuterated sample was produced. Under the experimental conditions used for NMR, however, the $^{15}$N, $^2$H double labelled sample showed apparent problems with poor quality spectra; there was a significant level of aggregation. This, along with the poor yield of deuterated protein produced, were responsible for the poor quality of the acquired spectrum. Further investigation of aggregation using size exclusion chromatography was attempted although the low level of protein concentration precluded these experiments to be performed with confidence.
Figure 3.18. 1D proton NMR spectrum of *A. gambiae* isolated FAD-binding domain. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8-298 K.
3.2.4.3.4 Soluble full length CPR

The *A. gambiae* full length CPR was produced with more success than the unstable FAD-binding domain. Like the FMN-binding domain, the diflavin reductase was expressed with high yields and showed a much higher level of stability than the FAD-binding domain. A 1D proton NMR spectrum was produced where clear, separate peaks were observed meaning the full length sample had been properly produced and was fully folded.

A $^{15}$N-labelled sample was produced and 2D spectrum generated (Figure 3.19). The $^1$H, $^{15}$N spectrum for *A. gambiae* CPR is a very crowded spectrum with many poorly-resolved resonances. Even the resolved resonances are overlapping. There are many regions where very weak peaks are present although the sensitivities remain poor due to rapid T$_2$ relaxation. This is characteristic of a very large protein, such as CPR. In order to alleviate the T$_2$ relaxation problem the protein sample was deuterated to produce a $^2$H, $^{15}$N sample.

Such techniques are commonly used in larger proteins like *A. gambiae* CPR which has a monomeric molecular mass of approximately 70 kDa, deuteration was essential in order to improve the quality of data. An obvious problem with producing deuterated samples in *E. coli* is that D$_2$O is not a good growth media for the bacteria
Figure 3.19. HSQC spectrum of $^1H^{15}N$, -labelled *A. gambiae* CPR. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8-298 K.
Growing the bacteria and expressing the protein in entirely D$_2$O based minimal media successfully produced a deuterated sample but there were some pitfalls to using this procedure. A D$_2$O based minimal media is a media consisting entirely of D$_2$O without any H$_2$O or additional rich media supplements. The growth rates of the bacterial cells were extremely slow compared to growth in the H$_2$O based minimal media. Such growth rates not only impact on time but also expose the bacteria to toxic D$_2$O for an extended period of time. This leads to slow growth but also an impaired yield which was another problem with a D$_2$O based media approach. The levels of protein were so low, <1 mg CPR per litre that the amount of D$_2$O required to produce a sample of adequate concentration was neither practical nor cost effective.

An alternative approach was clearly required in order to produce enough protein to generate a spectrum of any quality. Silantes (Silantes GmbH, München, Germany) provides specialised media for expression of isotope labelled protein samples. It is a type of rich media that vastly improves growth and expression conditions for *E. coli* when compared to standard D$_2$O-based media; thus protein samples can be produced which show high levels of labelled isotope incorporation without compromising on yields. As a trial of its efficacy, unlabelled Silantes was used in conjunction with D$_2$O based media to try to improve
growth rates and protein yields. This was indeed the case and when the D₂O media was spiked with 10 % unlabelled Silantes, the amount of protein produced increased to >10 mg per litre at a much more efficient rate. Predictably, the level of deuteration was impaired and the unlabelled Silantes element of the media contributed protons to the expressed protein.

Nevertheless, the ²H, ¹⁵N double labelled sample which was produced, despite being slightly deficient in ²H labelling, gave significantly better quality spectra than the singly labelled ¹⁵N-labelled protein sample (Figure 3.20). The regions of poor peak detection due to low signal-to-noise ratio in the protonated spectrum were much clearer in the deuterated spectrum. There were even several regions of peaks present in the deuterated spectrum that were completely missing from the protonated spectrum. In general, the deuterated spectrum was much neater with more peaks detected and those that were detected were much clearer and separate. Although this spectrum itself is not enough to generate any meaningful structural data, it confirms that recombinant *A. gambiae* CPR is folded. Nevertheless, the research demonstrates that it is possible and practical to prepare sufficient quantities of deuterated proteins for future NMR structure determination. This research establishes a protocol for making a deuterated sample, bearing in mind that potentially, there could have
Figure 3.20. HSQC spectrum of $^2$H, $^{15}$N-labelled *A. gambiae* CPR. Sample was prepared in 40 mM phosphate, 0.2 mM DTT; pH 6.8, 298 K
been some difficulties, such as cofactor incorporation and protein folding.

These data are extremely encouraging for a number of reasons. Firstly, this is the first NMR investigation on *A. gambiae* CPR and its constituent domains. All samples were effectively produced for such biophysical analysis, albeit with varying degrees of success. Production of deuterated protein samples can be very difficult to achieve and although the samples produced were not deuterated to a very high level, these samples show that deuteration is a very achievable possibility.

The levels of deuteration achieved in *A. gambiae* CPR, in this case, were assessed by interpretation of the 1D spectra. Figure 3.18 shows a typical 1D spectrum and such data can be utilised to make a rough assessment on the levels of deuteration in an analysed sample. In protein structure, the protons within NH groups (detected at p.p.m = ~9) are fully interchangeable with those in the purification buffers and thus will remain protonated even in a fully deuterated sample. Protons within CH₃ groups (detected at p.p.m = ~0) are, however, not interchangeable and once deuterated will not be re-protonated during purification. Since 1D spectra analyse protons, in a fully deuterated sample, the CH₃ peaks will disappear completely. In partially deuterated samples, the level is quantified by the relative peak height
of the CH$_3$ groups relative to the NH groups. If, for example, the NH peak had an arbitrary height of 3 and the CH$_3$ peak had an arbitrary height of 1, which suggests that 25% of the total peak height over those two groups was as a result of protons within CH$_3$ groups. If 25% of these hydrogen’s are protonated, this means that 75% are deuterated and that sample is believed to be approximately 75% deuterated.

In order to further continue the NMR study on *A. gambiae* CPR, time and, more importantly, money will need to be invested and fully labelled Silantes media used as the base media for sample expression. These data have provided essential groundwork to the *A. gambiae* NMR study and have suggested that introducing labelled Silantes, despite being an expensive medium, would potentially be a very worthwhile exercise in progressing structural determination of *A. gambiae* CPR.
3.2.5 Redox potentiometry

The tendency of a species to accept electrons and thus become reduced is measured by its reduction potential. The more positive the reduction potential, the more likely it is that the species will accept electrons and thus become reduced. Conversely, a more negative reduction potential increases the likelihood that a species will donate electrons and thus become oxidised. The transfer of electrons from one species to other is therefore strongly governed by the difference in reduction potentials of the two species.

In the case of CPR, the redox centres are the FMN and FAD cofactors and these are reduced by accepting electrons, donated as a hydride ion by NADPH. Each flavin accepts two electrons and therefore a number of redox couples are possible. In each case there are the oxidised/semiquinone (one electron reduced) couple and the semiquinone/hydroquinone (two electron reduced) couple. The calculated values for reduction potentials of these various couples can provide a valuable insight into the tendencies and properties of inter-flavin electron transfer within these enzymes. The reduction potentials of some CPR’s and related enzymes are shown (Figure 3.21).

Redox titrations were performed in a Belle Technology glove-box under a nitrogen atmosphere. All solutions were degassed under
Figure 3.21 The measured redox potentials of various diflavin reductases. The measured redox potentials of human CPR, rabbit CPR, P450 BM3 and the reductase domain of rat nNOS. The oxidised/semiquinone couples for FMN and FAD are shown in blue whilst the semiquinone/hydroquinone couples for FMN and FAD are shown in red. The dots are equivalent to the number of electrons: One for semiquinone species and two for hydroquinone species. The redox potential of the NADP$^+$/NADPH couple is shown as the black bar. Figure taken from Munro et al., 2001.
vacuum with Nitrogen prior to use in the glove-boxes. Oxygen levels were maintained at less than 2 p.p.m. Protein samples *A. gambiae* CPR, *A. gambiae* FAD and FMN-binding domains were applied through an anaerobic G25 Sephadex column (1 x 20 cm) (10DG Econo-Pac column, Bio-Rad) immediately on admission to the glove box to remove all traces of O₂, the column was pre-equilibrated and proteins buffer-exchanged into anaerobic buffer (100 mM potassium phosphate, 10 % (v/v) glycerol; pH 7.0) buffer. The protein solution (~15-30 μM in 5 mL buffer), was titrated electrochemically according to the method of Dutton (Dutton, 1978) using sodium dithionite as the reductant and potassium ferricyanide as the oxidant. 0.2 μL additions of dithionite and ferricyanide were made from concentrated stock solutions (typically 10-50 mM). Prior to titration, mediators were added to facilitate electrical communication between enzyme and electrode. Typically, 2 μM phenazine methosulfate (PMS) (E° +80 mV), 7 μM 2-hydroxy-1,4-naphthoquinone (HNQ) (E° -145 mV), 1 μM benzyl viologen (BV) (E° +311 mV), and 0.3 μM methyl viologen (MV) (E° +430 mV) were introduced to mediate within the full experimental range (between +100 to −480 mV) of the titration (Munro *et al.*, 2001; Ost *et al.*, 2001). At least 10-15 min was allowed to elapse between each addition of reductant/oxidant to ensure full equilibration of the solution and stabilisation of the electrode. Spectra were recorded over a range of 250-800 nm using a Cary UV-50 Bio UV-Visible
scanning spectrophotometer coupled to a fibre optic probe. The
electrochemical potential of the solution was measured using a Mettler
Toledo SevenEasy S20-K meter coupled to a Calomel electrode
(ThermoRussell Ltd.) at 25 °C. The electrode was calibrated using the
$\text{Fe}^{3+}/\text{Fe}^{2+}$ EDTA couple as a standard (+108 mV). The Calomel
electrode was corrected by +244 mV ± 2 mV, relative to the standard
(or normal) hydrogen electrode. Spectral data at wavelengths maximal
for the optical transitions, where appropriate, between oxidised
(quinone), 1-electron (semiquinone) and 2-electron reduced
(hydroquinone) forms of the flavin molecules were plotted versus the
applied potential, and the data fitted to the Nernst function using
Origin 7.0 (OriginLab, Northampton MA).

To ensure a good quality of redox titration experiment, spectra are
recorded in both oxidative and reductive directions. If the data coincide
with each other in both directions, it can be assumed the experimental
conditions were correct and the necessary equilibria were achieved. If,
however, the data do not coincide, hysteretical effects are seen
meaning the correct equilibria were not achieved under the
experimental conditions. Redox potentiometry experiments were
carried out using the specialised equipment at Manchester
Interdisciplinary Biocentre (MIB) with great assistance by Dr. Kirsty
McLean and Professor. Andy Munro.
Experiments were performed on each of the full length soluble A. gambiae CPR as well as the individual FMN and FAD-binding domains. Data for the individual FMN and FAD binding domains were fitted according to equation 1 which represents a two-electron redox process derived by extension to the Nernst equation and Beer-Lambert Law (Dutton, 1978; Daff et al., 1997.)

Eq 1.

\[
A = a \frac{10^{(E - E_{1'})/59} + b + c10^{(E_{2'} - E)/59}}{1 + 10^{(E - E_{1'})/59} + 10^{(E_{2'} - E)/59}}
\]

Equation 2 would be used to fit the data for the full length CPR and represents the sum of the two 2-electron redox processes (Dutton, 1978; Noble et al., 1999)

Eq. 2

\[
A = a10^{(E - E_{1'})/59} + b + c10^{(E_{2'} - E)/59} + \frac{d10^{(E - E_{3'})/59} + e + f10^{(E_{4'} - E)/59}}{1 + 10^{(E - E_{3'})/59} + 10^{(E_{4'} - E)/59}}
\]

Here, \( A \) is the total absorbance, \( a, b \) and \( c \) are component absorbance values contributed by one flavin in the oxidised, semiquinone and reduced states respectively, and \( d, e \) and \( f \) are the corresponding values from the second flavin. \( E \) is the observed potential, \( E_{1'} \) and \( E_{2'} \) are the midpoint potentials for the oxidised/semiquinone and
semiquinone/reduced couples, respectively, for the first flavin and \( E_{3} \)
and \( E_{4} \) are the corresponding midpoint potentials for the second flavin
(Munro et al., 2001)

Full analysis on both individual domains and the full length CPR was
not completed. The raw data was collected for all but the data not fully
fitted to the respective two- or four-electron reduced models in each
case. Although the diflavin reductase was stable in the reaction
conditions and the data acquired, the complex nature of the full length
CPR made fitting the data to the current model more difficult due to its
four electron reduction. The individual domain data is used to
overcome these complications and aid in fitting in the full length
enzyme. The FAD-domain was quite unstable in the reaction
conditions and the acquired data did not fit well into Nernst equation 1.
The FMN domain was more stable in the reaction conditions and the
data fitted well into Nernst equation 1. The spectrophotometric versus
applied potential values for this domain have been plotted at the
significant wavelengths.

3.2.5.1 FMN-binding domain

The spectral data for the A. gambiae FMN-binding domain (Figure
3.22) highlight a number of characteristic features of the domain under
redox conditions. The protein was stable during the entirety of the experimental procedure and good quality data was produced. Isosbestic points at 503 and 435 nm are generated and plot the transitions from oxidised/semiquinone through to semiquinone/hydroquinone species respectively by addition of the two electrons. The maximal absorbance for oxidised FMN-binding domain is at 454 nm and during reduction with the first electron, the absorbance intensity at this wavelength decreases. The decrease at 454 nm is coupled with an increase in intensity at 587 nm indicating semiquinone formation. As the domain is reduced further, the 454 nm peak continues to decrease as does the 587 nm peak. This shows that the semiquinone is being converted into the two-electron reduced hydroquinone form. The spectra show identical behaviour during both oxidative and reductive experiments (Figure 3.22)

Values of reduction potential for the one and two-electron reduced *A. gambiae* CPR FMN-binding domain were generated by plotting the potential versus absorbance at specific wavelengths. At 479 nm wavelength, there is the largest separation in oxidised versus reduced domain absorbancies (Figure 3.22) and when plotted, produces a double sigmoidal curve, each representing one of the two-electron reduction stages of the flavin.
Figure 3.22. Spectral properties of *A. gambiae* CPR FMN-binding domain during redox titrations. 30 μM *A. gambiae* CPR FMN-binding domain was first reduced using dithionite and re-oxidised with ferricyanide with spectra recorded at multiple time-points throughout. The isosbestic point at 503 nm, indicated by “a”, and is observed following one-electron reduction in the oxidised/semiquinone transition. The second isosbestic point at 435 nm, indicated by “b”, is observed following reduction by the second electron in the semiquinones/hydroquinone transition. Experiments were carried out with the help of Dr. Kirsty McLean (MIB).
The data were fitted using the two-electron Nernst equation and the midpoint potentials recorded. (Figure 3.23 A). The change in the absorbance at 587 nm follows the formation and subsequent decay of the semiquinone species following one then two electron reduction respectively. The separation of the midpoint potentials at this wavelength is indicative of the level of intensity of the semiquinone species. (Figure 3.23 B).

At the 435 nm wavelength, the isosbestic point for the second electron reduction step, any changes in absorbance will be during the first electron reduction step. Likewise at 503 nm, the isosbestic point for the first electron reduction step, any changes in absorbance will occur during the second electron reduction step. Thus, when absorbancies at the isosbestic points are plotted against the respective potentials, they provide a further level of comparison of calculated reduction potentials with respect to the data plotted at 479 and 587 nm. (Figure 3.24 A and B respectively).

The calculated midpoint potentials at 479 nm were -92 mV for the oxidised/semiquinone transition and -271 mV for the semiquinone/hydroquinone transition. These were in very good
Figure 3.23. Absorbance versus potential plots at 479 nm and 587 nm for *A. gambiae* CPR FMN-binding domain. A = The 479 nm wavelength represents the point at which there is the largest change in absorbance between the oxidised and reduced domain. B = The 587 nm wavelength plot represents the formation and decay of the semiquinone during one and two electron reduction respectively. Data was fitted using the two electron Nernst equation (eq1) by Dr. Kirsty McLean/Prof. Andy Munro (MIB).
Figure 3.24. Absorbance versus potential plots at 435 nm and 503 nm for A. gambiæe CPR FMN-binding domain. A = The 435 nm wavelength represents the isosbestic point for the semiquinone/hydroquinone transition. B = The 503 nm wavelength plot represents the isosbestic point for the oxidised/semiquinone transition. Data was fitted using the two electron Nernst equation (eq1) by Dr. Kirsty McLean/Prof. Andy Munro (MIB).
agreement with the calculated midpoint potentials measured at 587 nm which were -92 mV and -265 mV for the oxidised/semiquinone and semiquinone/hydroquinone transitions respectively. The reduction potentials measured at the isosbestic points corroborate these data and are calculated at -92 mV for the oxidised/semiquinone transition and -270 mV for the semiquinone/hydroquinone transition. The calculated reduction potential data for *A. gambiae* CPR FMN-binding domain are summarised in Table 3.2 with the human CPR FMN-binding domain data (Munro *et al.*, 2001) added for comparative purposes.

These data provide valuable information regarding the properties of flavin reduction of the FMN cofactor. These values are close to those seen in the human CPR FMN-binding domain (Table 3.2) with the major difference being a more negative $E$ value for the oxidised/semiquinone transition observed in *A. gambiae* CPR; -92 mV compared to -43 mV in human (Munro *et al.*, 2001). Known values for other diflavin reductases have been shown previously and the -92 mV observed in *A. gambiae* CPR is within the range of values observed for human and rabbit CPR’s (Figure 3.21). Without fully convoluted data for the FAD-binding domain and full length soluble CPR, conclusions regarding the entire protein cannot be drawn. However, this work on the isolated FMN-binding domain is good groundwork for redox
potentiometry in *A. gambiae* CPR and suggests a well produced FMN-binding domain.

### 3.5.4.2 FAD-binding domain

Although the data for the FAD-binding domain is yet to be fully deconvoluted, the raw data is presented (Figure 3.25). There is an isosbestic point at around 504 nm for the oxidised/semiquinone transition. The semiquinone/hydroquinone transition isosbestic point is less well defined but is possibly around 420 nm according to the raw data. The absorbance maxima for the oxidised FAD-binding domain are at 382 nm and 457 nm with the semiquinone absorbance maxima being 594 nm. These data are not too dissimilar to the FMN-binding domain and are also similar to values observed for the human FAD-binding domain (Munro *et al.*, 2001).
Table 3.2. Summary of the calculated reduction potentials in *A. gambiae* CPR FMN-binding domain. The $E$ values calculated following absorbance versus potential plots at different wavelengths are shown as well as the published values for human CPR FMN-binding domain. $^a$ = human FMN domain data from Munro *et al.*, 2001.

<table>
<thead>
<tr>
<th>Protein (wavelength)</th>
<th>Oxidised/semiquinone (mV)</th>
<th>Semiquinone/hydroquinone (mV)</th>
<th>Oxidised/reduced (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgFMN domain (479 nm)</td>
<td>-92 ± 1</td>
<td>-271 ± 5</td>
<td>-182 ± 3</td>
</tr>
<tr>
<td>AgFMN domain (587 nm)</td>
<td>-92 ± 1</td>
<td>-265 ± 2</td>
<td>-179 ± 2</td>
</tr>
<tr>
<td>AgFMN domain (435 nm)</td>
<td>-92 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AgFMN domain (503 nm)</td>
<td>-</td>
<td>-270 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>hFMN domain$^a$</td>
<td>-43 ± 7</td>
<td>-280 ± 8</td>
<td>-162 ± 8</td>
</tr>
</tbody>
</table>
Figure 3.25. Spectral properties of *A. gambiae* CPR FAD-binding domain during redox titrations. 30 µM *A. gambiae* CPR FAD-binding domain was first reduced using dithionite and re-oxidised with ferricyanide with spectra recorded at multiple time-points throughout. The isosbestic point at 504 nm, indicated by “a”, and is observed following one-electron reduction in the oxidised/semiquinone transition. The second isosbestic point is less clear but is apparently at around 420 nm, indicated by “b”, is observed following reduction by the second electron in the semiquinones/hydroquinone transition. Experiments were carried out with the help of Dr. Kirsty McLean (MIB).
3.3 Discussion

This chapter focussed on the expression, purification and initial characterisation of *A. gambiae* CPR and its isolated FMN and FAD-binding domains. The pET15b vector containing the N-terminal truncated soluble CPR (Dr. Mark Paine, Liverpool School of Tropical Medicine) was successfully expressed in competent BL-21 Codon Plus RP *E. coli* cells for optimal recombinant protein expression. Purified enzyme was correctly identified as *A. gambiae* CPR by mass spectrometry and was judged to be correctly folded by 1D proton NMR. Verification of the full length CPR was important considering the plasmid was to be used as a template for molecular dissection, using PCR, into the isolated FMN- and FAD-binding domains.

Initial manufacture of these domains into pET15b vectors subsequently proved unsuccessful for both domains. The need to remove an NdeI restriction site by incorporating a silent mutation into residue I617 proved a major stumbling block, a point emphasised by the failed sequencing of the FAD-binding domain plasmid. The FMN-binding domain plasmid was successfully made and sequenced however problems arose during expression and purification. A prominent double band was present following purification even following His-tag removal with the molecular weight difference between the two species remaining the same. Considering the supposed contaminant apparently
decreased in molecular weight following thrombin treatment, the most likely explanation is a C-terminal truncation of the FMN-binding domain. This, however, was not investigated further or verified by mass spectrometry as the requirement for both isolated binding domains necessitated new expression constructs to be made.

Subsequent molecular dissection, this time into the pETM-11 vector, of both the FMN and FAD-binding domains was much more successful. A method of alleviating the potential C-terminal truncation in the FMN-binding domain led to the manufacture of a domain which included an extension into the linker region. An extended FAD binding domain; extended at the N-terminus, was also made and following successful plasmid sequencing, these constructs were to be expressed, purified and characterisation. Both domains were successfully expressed in *E. coli* and purified without contaminating bands (Figures 3.7 and 3.9). Correct folding of both samples was confirmed by 1D proton NMR.

The redox potentials of human CPR along with its FMN and FAD-binding domains have previously been reported (Munro *et al.*, 2001). *A. gambiae* CPR, FMN and FAD-binding domains underwent identical anaerobic titrations in an attempt to establish the redox potentials of these enzymes. Experiments were performed successfully and raw data gathered. However, due to time constraints and instability issues with
the FAD-binding domain, full convolution of the raw data could not be fully undertaken. Analysis of the FMN-binding domain was, however, completed and a redox potential calculated. The oxidised/semiquinone transition in human FMN-binding domain was -43 mV, much more positive than the -92 mV calculated for *A. gambiae* FMN-binding domain (Table 3.2). This suggests that in the oxidised form, human FMN accepts electrons more readily than the *Anopheles* equivalent. One plausible explanation for this is that the FMN binds more weakly to the *A. gambiae* protein. The current data on the FMN domain only does not give much more information on potential mechanisms or major differences between the two CPRs, these can only be properly established when the FAD-binding domain and intact CPR data has been fully convoluted.

CPRs are known to form ‘air-stable’ semiquinones and following incubation with NADPH *A. gambiae* CPR produced a typical spectral pattern showing this enzyme conforms to conventional CPR behaviour. An interesting feature with regards to *A. gambiae* CPR characterisation was the apparent observed deficiency of incorporated FAD and to a greater extent FMN when compared to the human CPR (Table 3.1). This is particularly interesting considering that the same deficiency has been established in *A. minimus* CPR (Sarapusit *et al.*, 2008), a very closely related enzyme to *A. gambiae* CPR.
In the case of *A. minimus*, the deficiency was more obvious for both FMN and FAD. Residues in the FMN binding domain, F86 and F219, known to be involved in FMN stabilisation are conserved between *A. gambiae* and many other CPRs including rat and human; this is not so in *A. minimus* CPR, where both phenylalanines are replaced by leucines, this partially explaining its weak cofactor binding. Mutating these leucine residues to phenylalanine in *A. minimus* CPR brings FMN incorporation in-line with established human and rat CPR. It is, therefore, unclear as to why the FMN incorporation in *A. gambiae* is low considering the fact that phenylalanines are found in positions 86 and 219.

Sequence comparison alone is not sufficient, therefore, to fully understand the rationale for poor flavin incorporation. This was also apparent when using NMR; addition of exogenous FMN to $^{15}$N, $^{13}$C isotopically labelled samples yielded better spectra (Figure 3.15) The FMN-binding domain was sufficiently stable throughout for a good set of triple resonance data to be was acquired (Figures 3.16 and 3.17). However, the protein unfolded slowly over a two week period. The triple resonance data were of the required quality for assigning backbone resonances, however, time constraints precluded this from being performed. The FAD-binding domain was inherently unstable throughout the purification process and precipitation was a continual
problem, culminating in aggregation of samples in the magnet during data gathering. Because of its molecular weight, approximately 50 kDa, deuteration was required to improve peak sensitivity which was also the case for the full length CPR (Figure 3.20).

D$_2$O is not a good growth media for *E. coli*. The toxicity of D$_2$O allied to incredibly poor yields of pure protein meant an alternative labelling strategy was required. Unlabelled Silantes media was used to boost bacterial growth which subsequently impacted on yield by increasing it by at least 10-fold compared to D$_2$O media alone. Predictably, however, the preferential use of the Silantes rich media as a nutrient source led to isotopic dilution and a decrease in the level of $^2$H-labelling.

This is the first instance of NMR being used on *A. gambiae* CPR and its constituent FMN and FAD-binding domains. The biophysical data gathered in this thesis has provided a strong basis for future work of this kind. The FMN-binding domain has provided the most extensive data thus far; however, the successful production of isotopically labelled samples, including deuterated CPR and FAD-binding domain samples, lays the solid foundations for more comprehensive structural determinations of *A. gambiae* CPR in the future.
Chapter 4

4. NUCLEOTIDE AND FLAVIN COFACTOR BINDING

4.1 Introduction

NADPH binding and subsequent hydride interaction is a significant event in the activity of cytochrome P450 reductase (CPR). NADPH has been shown to bind in a bipartite fashion with initial interaction being at the 2’-phosphate moiety. Following 2’-phosphate interaction, the nicotinamide moiety is correctly positioned for full binding. Although the methods for nucleotide binding are well established, the relative binding affinities and energies for the different CPRs have not been thoroughly investigated. This is important if the differential behaviours of the CPRs from different organisms are to be exploited.

A common strategy in the purification of CPR’s is with the use of a 2’, 5’-ADP sepharose affinity chromatography resin. 2’, 5’-ADP is an analogue of NADPH and bound protein is eluted using 2’-AMP.
Previous work on *A. gambiae* CPR highlighted a very interesting observation in that the standard 2’, 5’-ADP sepharose failed to bind this enzyme; which was eluted in the flow through. The same resin had been successful in the processing of human CPR. This serendipitous observation suggested a significant difference in the binding characteristics between *A. gambiae* and human CPR’s to 2’, 5’-ADP. A comparative inhibition study using the same enzymes seemed to confirm this difference and suggested an approximate 10-fold weaker affinity of *A. gambiae* CPR for 2’, 5’-ADP when compared to the human equivalent.

In this research, Isothermal Titration Calorimetry (ITC) was used to assess the binding affinities and energies of both *A. gambiae* and human CPR to a number of NADPH nucleotide analogues (Figure 4.1). A similar study was previously reported for the human CPR (Grunau et al., 2006). Here, the experiments for human CPR were undertaken in parallel with the *A. gambiae* protein to ensure that the studies are undertaken under similar laboratory conditions, giving more confidence to any differences found. The crystal structure of the rat CPR showed that the NADPH-binding site is fully localised in the FAD-binding domain (Wang et al., 1997) and therefore, it is safe to assume that this will be the case for *A. gambiae* CPR. Using the expressed isolated *A. gambiae* FAD domain, binding characteristics
were generated for a selection of the tested analogues; these were then utilised as a further comparative aid for the activity of full-length *A. gambiae* CPR.
Figure 4.1. Structures of NADPH and related nucleotide ligands for ITC study. A = NADPH; B = NADP$^+$; C = NAD$^+$; D = 2', 5'-ADP; E = 2'-AMP; F = 3'-AMP.
4.2 Methodologies and Results

Isothermal Titration Calorimetry (ITC) is a technique used to ascertain thermodynamic parameters of chemical interactions. It is most commonly used to obtain thermodynamics information of interactions between small molecules, i.e. ligands, cofactors, metal ions, and larger macromolecules, i.e. proteins, DNA. With the current hardware, it is now also practical to use ITC for investigating protein-protein interactions. The quantitative measures that can be gained by ITC include binding stoichiometry (n), enthalpy change (ΔH), entropy change (ΔS) and binding affinity (K). From these, the measurement of the feasibility of reaction; the Gibbs free energy change (ΔG) can be calculated.

As two substances bind to each other, heat is either generated or absorbed in, respectively, an exothermic or endothermic reaction. Traditionally, a high concentration small molecule is injected sequentially into the lower concentration macromolecule. The heat exchange, either generated or absorbed, is measured over time by ITC. On an ITC isotherm, each peak is representative of the heat exchange following a single injection. The amount of heat exchange is proportional to the level of binding and as the reaction reaches saturation, the peak sizes get smaller meaning less binding is
occurring. The raw isotherm data is translated into a binding curve allowing thermodynamic parameters to be calculated.

The equipment used for Isothermal Titration Calorimetry measurements contain two cells- a reference and a sample cell. The reference cell, once filled with buffer or water, is not tampered with in order to avoid contamination. The sample cell is filled with the macromolecule and successively ligand is titrated into it, often in 1-2 µL injections. There are two thermostats in the system: the first maintains the cells at the experimental temperature by keeping them the same as the thermo-jacket. The second thermostat maintains the temperature difference between the reference and sample cell. This temperature difference needs to be kept constant and forms the basis for the measurement of heat exchange. As heat is absorbed or generated by addition of ligand into the sample cell, there is a change in temperature. Following the change in temperature upon binding, the power required to return to the initial inter-cell temperature difference is a direct measure of the total heat resulting from the reaction.

Appropriate buffer formulation is a major requirement in the design of ITC experiments. Certain substances should be not be used to avoid damaging the cell or having an effect on heat exchange. Phosphate based buffers are common; however, for ITC experiments involving CPR and associated enzymes, phosphate buffers are less than ideal.
The reason for this has been previously highlighted (Murataliev and Feyereisen, 2000; Grunau et al., 2006). Free phosphate binds to the NADPH binding site hence acting as a competitive inhibitor for nucleotide binding. Free phosphate has a $K_i$ of 33 mM in house fly reductase (Murataliev and Feyereisen, 2000) which is enough to cause a decrease in the binding affinities for nucleotide ligands. This was reported for human CPR, where binding of 2’, 5’-ADP to the enzyme had a 13-fold weaker affinity when ITC was performed in phosphate buffer as opposed to BES, a non-phosphate buffer (Grunau et al., 2006). For this reason, all of the ITC experiments performed in this study were carried out in a BES based buffer rather than one containing phosphate.

Purified protein samples were buffer-exchanged into ITC buffer (100 mM BES; pH 7.0) prior to experiments. This buffer was used for each experiment throughout the study. Unless freshly purified, samples were generally buffer-exchanged from 50 % glycerol stocks stored at -20 °C. Samples were centrifuged at 13,000 x g for 10 min and degassed at 23 °C (2 °C below the temperature at which the experiments were performed). All experiments were performed at 25 °C using the ITC200 calorimeter (Microcal) and data analysed using Origin 7.0 (Microcal). Typically, the protein concentration in the cell was in the region of 10-20 µM whilst different ligand concentrations in the syringe were tried
to elicit a binding curve. For the most part, ligand concentrations were in the range of 100-1000 µM.

4.2.1 Nucleotide binding affinities

One important role for CPR is to distinguish between nucleotides for effective electron transfer. Assessing the binding affinity of NADPH is difficult particularly since addition of NADPH to oxidised CPR will lead to a reduction event following hydride ion transfer from the nucleotide. When using ITC, this on-going process will produce inconsistent heat exchanges due to the presence of numerous species of both the enzyme and nucleotides present at any one time. This gives rise to poor quality binding curves. Furthermore, any binding affinity calculated cannot be attributed to any single species of enzyme or nucleotide. Ideally, therefore, in order to determine a binding affinity for NADPH, an inert analogue must be used. H₄NADP is such an analogue and will bind effectively to CPR without hydride ion transfer and hence electron transfer taking place. This keeps both the enzyme and nucleotide in the same oxidation state throughout the entirety of the experiment. The synthesis of H₄NADP is difficult, requires the use of specialised Palladium chemistry techniques and is beyond the scope of the current studies.
The study undertaken in this thesis focuses on understanding in greater depths, the role(s) of the phosphate groups in nicotinamide binding to CPR. This is done by comparing the interactions between NADP\(^+\) and NAD\(^+\).

NADPH and NADH differ only in the lack of a second phosphate group on the ribose ring in NADH (Figure 4.1). Nevertheless, clear differences in binding affinity between the two nucleotides have been widely reported. Since there is only one structural difference between these nucleotides, the additional phosphate group must be the major reason for this discrimination. Oxidised NADPH in the form of NADP\(^+\) does not fully interact with oxidised CPR due to the positively charged nicotinamide ring (Murataliev \textit{et al.}, 2000) however it is still able to bind weakly to the enzyme via phosphate group. Since binding still occurred using oxidised nucleotide, the binding affinities of NADP\(^+\) and the equivalent NAD\(^+\) were compared in both \textit{A. gambiae} and human CPR to confirm the importance of the phosphate group.
Figure 4.2. ITC Isotherms of NADP⁺ binding to *A. gambiae* CPR and isolated FAD-binding domain. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. A = NADP⁺ binding to *A. gambiae* CPR. NADP⁺ was prepared to 100 µM and injected into 10 µM *A. gambiae* CPR. B = NADP⁺ binding to *A. gambiae* FAD-binding domain. NADP⁺ was prepared to 100 µM and injected into 10 µM *A. gambiae* FAD-binding domain.
The $K_d$ of \textit{A. gambiae} CPR for NADP$^+$ was 363 nM whilst for human CPR the $K_d$ was determined to be 69 nM. This is an approximate 5-fold stronger affinity for NADP$^+$ in human CPR than is determined for \textit{A. gambiae} CPR. The binding affinity of the \textit{A. gambiae} FAD-binding domain alone for NADP$^+$ was also investigated and in this case, the $K_d$ was found to be 292 nM. This value for the isolated FAD-binding domain is similar to the observed binding affinity for the full length \textit{A. gambiae} CPR (Figure 4.2) implying that the nucleotide-binding site is fully localised within the FAD-binding domain.

In considering the binding affinity of NAD$^+$ to all three enzymes, an exact $K_d$ value couldn’t be calculated under the chosen experimental conditions. This was due to the extremely weak affinities for NAD$^+$. Although an exact value could not be established, the estimate is that the binding is at least an order of magnitude weaker than those observed for NADP$^+$. The sheer deficiency in binding of NAD$^+$ can be easily observed when the binding curves for NADP$^+$ and NAD$^+$ are overlaid (Figure 4.3). This confirms an absolute necessity for the presence of a phosphate group on the ribose ring for efficient binding of NADPH nucleotides to CPR.
Figure 4.3. ITC Isotherms of NADP\(^+\) binding to human CPR and A. gambiae CPR overlaid with NAD\(^+\) binding to the same samples. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. In each case, blue isotherm = NADP\(^+\), red isotherm = NAD\(^+\) A = NADP\(^+\) binding to human CPR overlaid with NAD\(^+\) binding to human CPR. NADP\(^+\) and NAD\(^+\) were prepared to 100 µM and injected into 10 µM A. gambiae CPR. B = NADP\(^+\) binding to A. gambiae CPR overlaid with NAD\(^+\) binding to A. gambiae CPR. NADP\(^+\) and NAD\(^+\) were prepared to 100 µM and injected into 10 µM A. gambiae FAD-binding domain.
4.2.2 2’phosphate binding affinities

Initial recognition of NADPH by CPR is at the 2’-phosphate moiety and this binding was assessed in both *A. gambiae* and human CPR’s. The importance of the 2’-phosphate group in nucleotide binding was highlighted by comparison of 2’-AMP and 3’-AMP nucleotides whilst the synergistic interaction involving a second 5’-phosphate was shown by comparison between 2’-AMP and 2’,5’-ADP binding.

2’,5’-ADP is the phosphate derived nucleotide analogue most representative of the NADPH molecule since it contains both phosphate groups attached in the appropriate positions. It is also the base molecule for the resin used in the purification of CPR’s. As mentioned earlier, previous studies suggested a large disparity in the respective 2’, 5’-ADP binding affinities for *A. gambiae* and human CPR. ITC data was generated for this interaction to corroborate this previous observation. The calculated $K_d$ for the interaction between 2’, 5’-ADP and *A. gambiae* CPR was 410 nM. The binding affinity for 2’, 5’-ADP with human CPR was much stronger at 38 nM; this is an approximately 10-fold stronger affinity than what was observed with *A. gambiae* CPR. The binding affinity of the isolated *A. gambiae* FAD-binding domain for 2’, 5’-ADP was also investigated and the $K_d$ for
Figure 4.4. ITC Isotherms of 2', 5'-ADP binding to *A. gambiae* CPR, human CPR and isolated *A. gambiae* FAD-binding domain. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. 

**A** = 2', 5'-ADP binding to *A. gambiae* CPR. 2', 5'-ADP was prepared to 200 µM and injected into 10 µM *A. gambiae* CPR.

**B** = 2', 5'-ADP binding to human CPR. 2', 5'-ADP was prepared to 50 µM and injected into 10 µM human CPR.

**C** = 2', 5'-ADP binding to *A. gambiae* isolated FAD-binding domain. 2', 5'-ADP was prepared to 100 µM and injected into 10 µM *A. gambiae* isolated FAD-binding domain.
this interaction was calculated at 322 nM. This was very similar to what was observed for the full length *A. gambiae* CPR which is expected if the binding site is fully localised within the FAD-binding domain (Figure 4.4). These data do indeed corroborate what was suggested using inhibition experiments and provide a reasonable explanation for the inability to purify *A. gambiae* CPR using 2’,5’-ADP sepharose resin.

When 2’-AMP was bound to *A. gambiae*, the generated curve showed clear binding of nucleotide to protein. From the binding isotherm, the calculated *K*<sub>d</sub> for this interaction was 4 µM. Binding of 2’-AMP to human CPR was also assessed using ITC. The binding isotherm looked a lot better than the *A. gambiae* CPR curve with a larger number of points located at the lower end of the sigmoid and the *K*<sub>d</sub> was calculated to be 1.6 µM; this is approximately 3-fold stronger affinity than the affinity for seen in *A. gambiae* CPR (Figure 4.5). These values are in the µM range whilst the *K*<sub>d</sub> values for 2’,5’-ADP binding are in the nM range showing that the inclusion of the 5’-phosphate to the nucleotide definitively increases affinity by stabilising the interaction.

3’-AMP binding was analysed to confirm the importance of the 2’-phosphate to nucleotide interaction with CPR. Under the experimental conditions, no reliable *K*<sub>d</sub> could be obtained showing that the binding
Figure 4.5. ITC Isotherms of 2'-AMP binding to *A. gambiae* CPR and human CPR. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. A = 2'-AMP binding to human CPR. 2'-AMP was prepared to 500 µM and injected into 10 µM human CPR. B = 2'-AMP binding to *A. gambiae* CPR. 2'-AMP was prepared to 500 µM and injected into 10 µM *A. gambiae* CPR.
to 3’-AMP was too weak to be determined by ITC. This is the case for both human and \textit{A. gambiae} CPR.

In contrast, human CPR has stronger binding affinity than \textit{A. gambiae} CPR for both \textit{2’-AMP} and \textit{2’, 5’-ADP}. Even though a figure could not be placed on 3’-AMP binding affinity, what becomes clear is that replacing the 2’-phosphate leads to a dramatic decrease in binding affinity. This illustrates the specificity of recognition of the 2’-phosphate moiety in NADPH binding. A clear indication of the weakness of 3’-AMP binding is shown when the binding curve is overlaid with the 2’, 5’-ADP binding curves for both \textit{A. gambiae} and human CPR (Figure 4.6).

The $K_d$ values observed for NADP$^+$ or 2’, 5’-ADP were, respectively, 363 nM and 410 nM respectively for \textit{A. gambiae}, CPR, and 69 nM and 38 nM for human CPR. For the \textit{A. gambiae} FAD-binding domain these values were, respectively for NADP$^+$ and 2’, 5’-ADP, 292 nM and 322 nM. The results emphasise the fact that NADP$^+$ does not fully interact with CPR and that the binding affinity is, in the most part, almost entirely down to 2’-phosphate and 5’-phosphate recognition rather than nicotinamide binding.
Figure 4.6. ITC Isotherms of 2', 5'-ADP binding to human CPR and A. gambiae CPR overlaid with 3'-AMP binding to the same samples. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. A = 2', 5'-ADP binding to A. gambiae CPR overlaid with 3'-AMP binding to A. gambiae CPR. 2',5'-ADP was prepared to 200 µM and 3'-AMP was prepared to 2 mM and injected into 10 µM A. gambiae CPR blue isotherm = 2',5'-ADP, black isotherm = 3'-AMP. B = 2', 5'-ADP binding to human CPR overlaid with 3'-AMP binding to human CPR. 2',5'-ADP was prepared to 50 µM and 3'-AMP was prepared to 1 mM and injected into 10 µM human CPR. Red isotherm = 2', 5'-ADP, black isotherm = 3'-AMP.
4.2.3 Analysis of thermodynamic data

Analysis of the binding affinity alone is not enough to fully ascertain the characteristics of nucleotide interaction. Thermodynamic properties give an idea of the likelihood of binding events and assess their energetic favourability. Enthalpy change ($\Delta H$) is a measure of the change in heat energy during the progression of a reaction. In an endothermic reaction, the amount of heat energy at the end of a reaction is higher than at the beginning; hence heat is absorbed leading to a positive $\Delta H$. Conversely, in an exothermic reaction, the amount of heat energy at the end of a reaction is lower than the beginning. Heat is therefore lost during the reaction leading to a negative $\Delta H$.

Entropy change ($\Delta S$) is another measurement taken into account during thermodynamic characterisation. The entropy of a reaction is loosely defined as the tendency of a reaction to become more disordered and can be a measure of the spontaneity of a reaction to take place. In general, a positive entropy, i.e. tending towards disorder, makes a reaction spontaneous whereas a negative entropy, i.e. tending towards order, require more energy input and hence is less spontaneous. However, in a closed system, certain spontaneous reactions can have negative $\Delta S$ as it is a measure of the energy gained and lost by both the system and its surroundings. The system can have negative entropy
however, when the energy lost to the surroundings is considered, the total entropy is positive which signifies a spontaneous process.

In closed thermodynamic systems which operate at constant temperature and pressure, an easier and more effective measure of the spontaneity of a reaction to take place is to consider the Gibbs free energy change ($\Delta G$) of a reaction. $\Delta G$ is calculated by considering $\Delta H$ and $\Delta S$ as well as the temperature in the following equation: $\Delta G = \Delta H - T\Delta S$. A positive $\Delta G$ means a reaction will not take place at all, whilst a negative $\Delta G$ means a reaction will take place spontaneously. A reaction with a negative $\Delta G$ may be spontaneous but immeasurably slow, meaning the reaction effectively does not take place. Such reactions, however, may be made more likely by use of a catalyst to lower the activation energy barrier. $\Delta G$ is, therefore, purely a thermodynamic measure of feasibility and can be applied to suggest the likelihood of a reaction occurring.

The thermodynamic data discussed in this section is summarised in Tables 4.1, 4.2 and 4.3. The measured $\Delta H$ for human CPR binding to 2’, 5’-ADP was -20.36 kcal mol$^{-1}$ which is quite a large exothermic reaction. This is much larger than what was observed for A. gambiae CPR binding to 2’, 5’-ADP where the measured $\Delta H$ was -13.13 kcal mol$^{-1}$. The much larger negative $\Delta H$ observed in human CPR suggests more highly favourable binding than when A. gambiae CPR is bound.
to this ligand. Although 2’-AMP has a much lower binding affinity than 2’, 5’-ADP in both human and *A. gambiae* CPR, the same observation can be seen with respect to $\Delta H$. The $\Delta H$ was a lot less negative when 2’-AMP is the ligand in both enzymes suggesting less favourable binding characteristics. When NADP$^+$ was the ligand, the same pattern is observed when comparing human and *A. gambiae* CPR’s. The $\Delta H$ for NADP$^+$ binding to human CPR was -19.46 kcal mol$^{-1}$ whilst in binding to *A. gambiae* CPR the $\Delta H$ was less negative at -11.97 kcal mol$^{-1}$. The fact that in both human and *A. gambiae* CPRs the $\Delta H$ for NADP$^+$ and 2’, 5’-ADP binding were very similar further implies that most of the energy in NADPH is drawn from the 2’-phosphate interaction with very little being drawn from nicotinamide interaction.

The $\Delta G$ is a measure of the likelihood of the reaction to take place. When analysing the $\Delta G$ of each interaction, it becomes clear that there are differences in binding of NADPH nucleotides to human and *A. gambiae* CPR’s. The $\Delta G$ for 2’, 5’-ADP binding to human CPR was measured at -10.39 kcal mol$^{-1}$ whereas the same ligand binding to *A. gambiae* CPR was -8.40 kcal mol$^{-1}$. This difference, allied to the $\Delta H$ and $K_d$ strongly suggest a stronger and much more favourable interaction between 2’, 5’-ADP and human CPR than with *A. gambiae* CPR. These observations were very similar when NADP$^+$ was the
ligand used. The $\Delta G$ for NADP$^+$ binding to human CPR was -10.55 kcal mol$^{-1}$ and the $\Delta G$ for binding to A. gambiae was lower at -8.73 kcal mol$^{-1}$. Since most of the binding energy in NADPH binding is derived from 2'-phosphate interaction, these observations in 2', 5'-ADP and NADP$^+$ binding can be transferred to what may be observed when native NADPH binds to CPR.

When comparing the isolated A. gambiae FAD-binding domain to the full length enzyme, some interesting points can be drawn. The $\Delta G$ values for the isolated FAD-binding domain are -8.48 kcal mol$^{-1}$ and -9.07 kcal mol$^{-1}$ 2', 5'-ADP and NADP$^+$ respectively. These are very similar to what was observed in the full length A. gambiae CPR. The biggest difference however was seen in the $\Delta S$ values. The entropy changes were much more favourable for both 2',5'-ADP and NADP$^+$ in the full length A. gambiae CPR than the isolated A. gambiae FAD-binding domain; -4.73 kcal mol$^{-1}$ and -3.24 kcal mol$^{-1}$ for 2',5'-ADP and NADP$^+$ respectively with A. gambiae CPR and -5.30 kcal mol$^{-1}$ and -6.54 kcal mol$^{-1}$ for 2',5'-ADP and NADP respectively with isolated A. gambiae FAD-binding domain. In the three-dimensional structure of the rat CPR, following NADPH binding, the FMN-binding domain is aligned in such as way that the FMN cofactor is in fact spatially close to the FAD and NADPH binding sites (Wang et al., 1997) and these data suggest that the very presence of the FMN-
binding domain in the full length protein potentially affects the entropic favourability and NADPH binding.
Table 4.1. Measured thermodynamic parameters for *A. gambiae* CPR interaction with a selection of nucleotide ligands. Experiments were performed at 25 °C in 100 mM BES; pH 7.0. The quoted errors are standard errors derived from duplicate experiments. $n$ = binding stoichiometry, $K_d = 1/K_{obs}$.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$n$</th>
<th>$K_{obs}$ (x 10$^5$ M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$T\Delta S$ (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP$^+$</td>
<td>1.00 ± 0.04</td>
<td>27.5 ± 1.29</td>
<td>363 ± 17</td>
<td>-11.97 ± 0.64</td>
<td>-3.24</td>
<td>-8.73</td>
</tr>
<tr>
<td>2',5'-ADP</td>
<td>1.01 ± 0.03</td>
<td>24.4 ± 1.07</td>
<td>410 ± 18</td>
<td>-13.13 ± 0.35</td>
<td>-4.73</td>
<td>-8.40</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>1.01 ± 0.22</td>
<td>2.51 ± 0.11</td>
<td>4000 ± 175</td>
<td>-7.66 ± 1.98</td>
<td>-2.19</td>
<td>-5.47</td>
</tr>
<tr>
<td>3'-AMP</td>
<td>results not recorded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>results not recorded</td>
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<td></td>
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</tr>
</tbody>
</table>
Table 4.2. Measured thermodynamic parameters for human CPR interaction with a selection of nucleotide ligands. Experiments were performed at 25 °C in 100 mM BES; pH 7.0. The quoted errors are standard errors derived from duplicate experiments. \( n \) = binding stoichiometry, \( K_d = 1/K_{obs} \).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( n )</th>
<th>( K_{obs} \times 10^5 \text{ M}^{-1} )</th>
<th>( K_d ) (nM)</th>
<th>( \Delta H ) (kcal mol(^{-1} ))</th>
<th>( T\Delta S ) (kcal mol(^{-1} ))</th>
<th>( \Delta G ) (kcal mol(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP(^+)</td>
<td>1.01 ± 0.12</td>
<td>145 ± 24.5</td>
<td>69 ± 12</td>
<td>-19.46 ± 2.92</td>
<td>-8.91</td>
<td>-10.55</td>
</tr>
<tr>
<td>2',5'-ADP</td>
<td>1.00 ± 0.02</td>
<td>263 ± 26.3</td>
<td>38 ± 3.8</td>
<td>-20.36 ± 0.53</td>
<td>-9.97</td>
<td>-10.39</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>1.02 ± 0.06</td>
<td>6.15 ± 1.69</td>
<td>1600 ± 440</td>
<td>-11.07 ± 0.88</td>
<td>-3.21</td>
<td>-7.86</td>
</tr>
<tr>
<td>3'-AMP</td>
<td></td>
<td></td>
<td></td>
<td>results not recorded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD(^+)</td>
<td></td>
<td></td>
<td></td>
<td>results not recorded</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3. Measured thermodynamic parameters for *A. gambiae* isolated FAD-binding domain interaction with a selection of nucleotide ligands. Experiments were performed at 25 °C in 100 mM BES; pH 7.0. The quoted errors are standard errors derived from duplicate experiments. *n* = binding stoichiometry, *KD* = 1/*Kobs*.

<table>
<thead>
<tr>
<th>Ligand</th>
<th><em>n</em></th>
<th><em>Kobs</em> (x 10^2 M⁻¹)</th>
<th><em>Kd</em> (nM)</th>
<th>Δ<em>H</em> (kcal mol⁻¹)</th>
<th>TΔ<em>S</em> (kcal mol⁻¹)</th>
<th>Δ<em>G</em> (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP⁺</td>
<td>0.99 ± 0.03</td>
<td>37.7 ± 1.49</td>
<td>265 ± 10</td>
<td>-15.61 ± 0.49</td>
<td>-6.54</td>
<td>-9.07</td>
</tr>
<tr>
<td>2’,5’-ADP</td>
<td>0.99 ± 0.05</td>
<td>31.1 ± 1.80</td>
<td>322 ± 19</td>
<td>-13.78 ± 0.75</td>
<td>-5.30</td>
<td>-8.48</td>
</tr>
<tr>
<td>2’-AMP</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>results not recorded</td>
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<tr>
<td>3’-AMP</td>
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<td>results not recorded</td>
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<tr>
<td>NAD⁺</td>
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<td>results not recorded</td>
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</tbody>
</table>
4.2.4 FMN binding analysis

ITC was also utilised to assess the binding respective binding affinity of FMN to the isolated FMN-binding domain of both *A. gambiae* and human CPR. It was shown previously that fully purified *A. gambiae* CPR has a deficiency in FMN when compared to the human enzyme and the possibility that this was down to a lower flavin cofactor affinity in the *Anopheles* enzyme was investigated. The apo-FMN binding domain was created in each case by the removal of FMN cofactor from purified domain.

Flavins were removed to generate apo-protein by modification of the method of Edmondson and Tollin, 1971 (Barsukov *et al.*, 1997). For precipitation, protein samples were prepared to a concentration of 0.5 mg/mL in 40 mM phosphate, 0.2 mM DTT, 0.02 mM EDTA, 20 % glycerol; pH 6.8. A 30 % (w/v) solution of trichloroacetic acid was added, in the dark at 4 °C, to the protein samples. to a final concentration of 3 %. The samples were centrifuged at 20,000 x g for 5 min at 4 °C and the pellet washed with ice-cold 3 % trichloroacetic acid, 1.3 mM DTT in distilled H₂O. The sample was centrifuged for a further 5 min at 20,000 x g at 4 °C and the pellets re-suspended in 100 mM BES; pH 7.0 for ITC experimentation.
The apo-FMN-binding domains from both the human and *A. gambiae* CPR were stable at high concentrations. The binding isotherms for both the apo-human and apo-*A. gambiae* FMN-binding domains are shown (Figure 4.7). Both isotherms show reasonable binding and saturation of the apo-proteins is achieved with excess flavin. The $K_d$ for FMN binding to the isolated apo-human FMN-binding domain was calculated to be 23 nM which is almost 4-fold stronger than for the isolated apo-*A. gambiae* FMN-binding domain which was calculated to be 83 nM.

The thermodynamic characteristics also heavily favoured FMN binding to the human FMN-binding domain over the *A. gambiae* FMN-binding domain. The $\Delta H$ for FMN binding to the human FMN-binding domain was -19.46 kcal mol$^{-1}$ which is seemingly more favourable than the -13.18 kcal mol$^{-1}$ which was measured for FMN binding to the *A. gambiae* FMN-binding domain. The $\Delta G$ for FMN binding to the human FMN-binding domain was -10.28 kcal mol$^{-1}$ suggesting the binding of FMN flavin to human FMN-binding domain is more favourable than to *A. gambiae* FMN-binding domain which had a calculated $\Delta G$ of -9.60 kcal mol$^{-1}$. The thermodynamic parameters for FMN binding is summarised in Table 4.4.
Figure 4.7. ITC Isotherms of FMN binding to *A. gambiae* and human isolated apo-FMN-binding domains. Samples and ligands were prepared in 100 mM BES; pH 7.0. Experiments were performed at 25 °C. **A** = FMN binding to *A. gambiae* apo-FMN binding domain. FMN was prepared to 300 µM and injected into 30 µM *A. gambiae* apo-FMN binding domain. **B** = FMN binding to human apo-FMN binding domain. FMN was prepared to 200 µM and injected into 30 µM human apo-FMN binding domain.
To fully assess how these data were represented in the full length enzyme, generation of the apo-full length enzyme would be ideal. This, however, proved fairly difficult as once flavins were stripped off, the apo-CPRs were unstable and prone to precipitation. This is possibly due to the influence of the FAD-binding domain. The apo-FAD-binding domain from *A. gambiae* CPR, when expressed and purified separately, was very unstable, with high levels of precipitation. It is possible that this highly unstable apo-form may have a significant contribution to the instability of the full length apo-CPR.
Table 4.4. Measured thermodynamic parameters for *A. gambiae* and human CPR interaction with FMN. Experiments were performed at 25 °C in 100 mM BES; pH 7.0. $n = $ binding stoichiometry, $K_d = 1/K_{obs}$.

<table>
<thead>
<tr>
<th>apo-FMN domain</th>
<th>$n$</th>
<th>$K_{obs}$ ($\times 10^5$ M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$T\Delta S$ (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>1.00 ± 0.01</td>
<td>438 ± 17.5</td>
<td>23 ± 0.9</td>
<td>-19.46 ± 0.35</td>
<td>-9.18</td>
<td>-10.28</td>
</tr>
<tr>
<td><em>A. gambiae</em></td>
<td>0.98 ± 0.01</td>
<td>120 ± 3.19</td>
<td>83 ± 2.0</td>
<td>-13.18 ± 0.19</td>
<td>-3.58</td>
<td>-9.60</td>
</tr>
</tbody>
</table>
4.3 Discussion

ITC was used to probe the NADPH-binding site of *A. gambiae* CPR. A similar investigation has previously been performed on human CPR (Grunau *et al.*, 2006). In the Grunau *et al.* study, a VP-ITC (MicroCal) calorimeter was used. In the current studies, a more recent model of the ITC equipment, an ITC200 (MicroCal) calorimeter was used. In both these studies, the results for human CPR are comparable. The binding of FMN to the apo-isolated FMN binding domain of both human and *A. gambiae* CPR was also investigated and showed a marked difference in FMN binding affinity for both enzymes.

The FMN-binding affinity of the apo-human FMN-binding domain was much stronger than was observed for the apo-*A. gambiae* FMN-binding domain (Table 4.4). *A. gambiae* CPR shows a distinct deficiency of FMN in the purified enzyme, even when supposedly fully saturated with flavin. This lack of fully incorporated flavin is not common to *A. gambiae* as *A. minimus* has recently been shown to also be flavin deficient when compared to mammalian CPRs (Sarapusit *et al.*, 2008). The observation that FMN has lower binding affinity, and less thermodynamically favourable binding parameters, to *A. gambiae* CPR than the human equivalent provides a strong evidence to corroborate this finding.
The three-dimensional structures of the rat (Wang et al., 1997) and yeast (Lamb et al., 2006) CPR and the rat nNOS FAD domain (Zhang et al., 2001) have collectively provided details of the cofactor binding sites for the oxidoreductases. FMN binds in the region \textsuperscript{139}TYGE\textsuperscript{PD} and \textsuperscript{175}NKTYEHF\textsuperscript{N} (rat CPR numbering), with Y\textsuperscript{140} and Y\textsuperscript{178} sandwiching the FMN isoalloxazine ring, and F\textsuperscript{181} stabilising the cofactor binding (Paine et al., 2001). Figure 4.8 shows a multiple sequence alignment of \textit{A. gambiae} CPR with \textit{A. minimus} CPR, rat CPR, \textit{S. cerevisiae} CPR and rat nNOS reductase domain. As far as \textit{A. gambiae} CPR is concerned, the residues mentioned above are all conserved with the exception of F\textsuperscript{181} (in rat CPR) replaced by Y\textsuperscript{184} (in \textit{A. gambiae} CPR). However, it has previously been reported that mutation of F\textsuperscript{181} to Y\textsuperscript{181} in human CPR had minimal effects on FMN binding and enzymatic activity (Paine et al., 2001); hence, replacing the tyrosine residue in \textit{A. gambiae} CPR protein is of little consequence for FMN affinity. Therefore, despite the availability of good structures of homologous proteins, these have so far proved inadequate for understanding the cofactor binding characteristics of the \textit{A. gambiae} CPR.
Figure 4.8. Multiple sequence alignments of the FMN binding region of *A. gambiae* CPR, *A. minimus* CPR, rat CPR (rCPR), *S. cerevisiae* CPR (yCPR) and the reductase domain of rat nNOS (nNOS). Residues involved in FMN binding (grey) and stabilisation (red) are highlighted. Sequences were aligned using ClustalW.
A clear and obvious difference between the human and *A. gambiae* CPR’s is in the binding affinities of NADPH nucleotide analogues; 2’, 5’-ADP, 2’-AMP and NADP+. These corroborate previous inhibition studies performed on these enzymes which suggested an approximate 10-fold decreased affinity for 2’, 5’-ADP in *A. gambiae* CPR when compared to human CPR. ITC data suggested this to be the case and the relative weak affinity to NADPH analogues extended to 2’-AMP and NADP+ (Tables 4.1 and 4.2).

When the rat CPR crystal structure is compared the modelled *A. gambiae* CPR (provided by Dr. Dan Rigden, School of Biological Sciences, University of Liverpool) there is an apparent difference in the residues interacting with the 2’,5’-ADP group. In rat and human CPR, residue 606 is a glutamine whilst the corresponding residue 607 in *A. gambiae* is a threonine. On further inspection, this provides a clear structural difference in this region. In rat CPR, the side chain of Q606 forms the face of a cavity and is approximately 3 Å away from the adenine ring of NADPH, close enough to form a hydrogen bond. In contrast the distance between the adenine ring and the T607 side chain in *A. gambiae* CPR is almost 6 Å; too far to form a hydrogen bond (Figure 4.9).

This lack of additional stabilisation in *A. gambiae* CPR could possibly be a cause for low 2’, 5’-ADP binding affinity in this enzyme. The *A.
*gambiae* T607Q mutant (Dr. Mark Paine, Liverpool School of Tropical Medicine) was generated in an attempt to alter the stabilisation of 2’,5’-ADP binding in *A. gambiae* CPR towards that of the rat enzyme. This binding was analysed by ITC (Figure 4.10); however, the $K_d$ of the mutant for 2’, 5’-ADP was near identical to that of the wild-type *A. gambiae* CPR.

Thermodynamic parameters were also measured and difference in $\Delta H$ and $\Delta G$ values further point towards a potential difference in nucleotide binding energetics as well as affinities. In human CPR-nucleotide binding events, a more negative $\Delta G$ combined with a more negative $\Delta H$ indicate these interactions occur more readily and are thermodynamically more favourable than *A. gambiae* CPR-nucleotide interactions. An interesting observation involves the differences in $T\Delta S$ values of human and *A. gambiae* CPR. The entropies of human CPR binding events appear less favourable, i.e. more negative and hence tending more towards order, than the *A. gambiae* reactions. This apparent unfavourability however is counter balanced by much more favourable $\Delta H$ values making the overall energy value, $\Delta G$, more favourable (Tables 4.1 and 4.2).
Figure 4.9. Comparison of NADPH interaction with side chains of Q606 in rat and T607 in A. gambiae CPR. A = Interaction of NADPH (red) with the Q606 side chain (blue) in rat CPR (PDB – 1AMO). B = Interaction of NADPH (red) with the T607 side chain (blue) in A. gambiae CPR model (Dr. Dan Rigden). In each case, the figures were generated using PyMol and the distances are shown in Å.
Figure 4.10. ITC Isotherms of 2’, 5’-ADP binding to *A. gambiae* CPR T607Q mutant. Sample and ligand were prepared in 100 mM BES; pH 7.0. Experiments were run at 25 °C. 2’, 5’-ADP was prepared to 200 µM and injected into 10 µM *A. gambiae* CPR.
The binding of the isolated *A. gambiae* FAD-binding domain was assessed using certain ligands. It was obvious that although the binding affinities and overall free energies were essentially similar to the full length *A. gambiae* CPR, the specific energetics of binding were a little different. The entropy changes, $T\Delta S$, were more favourable in the full length CPR than the FAD-binding domain (Tables 4.1 and 4.3). This suggests the presence of the FMN-binding domain can influence NADPH binding.

In the three-dimensional structure of the rat CPR, following NADPH binding, the FMN domain is aligned in such a way that the FMN cofactor is in fact spatially close to the FAD and NADPH binding sites. This observation concurs with all the known structures of CPRs whose ‘bowl’ shapes bring the FMN and FAD domain in close proximity (Figure 1.10, section 1.6.6); in fact the FMN is 3.5Å from the FAD at the closest point (Wang *et al.*, 1997). The model of the *A. gambiae* CPR and this structure demonstrates a similar trait with regards to FMN and FAD position within the NADPH-bound enzyme (Figure 4.11). In this case, the FMN and FAD-cofactors were 3.8Å at the C₈M atoms and 4.3Å at the C₇M atoms of the respective xylene rings.

Upon NADPH binding, the flexibility of CPR allows the FMN-domain to be brought closer to the FAD and NADPH binding sites. This
Figure 4.11. Proximity of FMN and FAD cofactors in NADPH-bound *A. gambiae* CPR modelled structure. **A** = The proximity of the C₈M atoms on the xylene rings. **B** = The proximity of the C₇M atoms on the xylene rings. *Green* = FMN; *Red* = FAD; *Blue* = NADPH. Distances are expressed in angstroms. The model was built by Dr. Dan Rigden (University of Liverpool, School of Biological Sciences) and the figure generated using PyMol.
conformational shift could account for the thermodynamic differences observed between the full length CPR and the isolated FAD-binding domain. In addition to the full length CPR, the binding of both 2’, 5’-ADP and NADP+ to the FAD-binding domain was investigated. For both the CPR and the FAD-binding domain, the ΔG value for each ligand was quite similar (Tables 4.1 and 4.3). Despite this near identical overall energy, there are more clear discrepancies in the ΔH and TΔS values in each case. There is consistently a more favourable ΔH value observed in ligand binding to the FAD-binding domain, however, this is counteracted by a much more favourable TΔS value in binding to CPR.

In exothermic reactions, the ΔH is a measurement of the energy released to the surroundings, thus in binding to the FAD-binding domain, more energy is released. This is potentially due to the fact that in the full length CPR, which includes the FMN binding domain, the act of nucleotide binding necessitates a conformational change to bring the FMN cofactor in close proximity to the FAD and NADPH. It is possible; therefore, that some of the binding energy is expended in shifting the FMN-binding domain in full length CPR. This is not an issue in the case of the isolated-binding domain and could explain the more favourable ΔH values observed in this case. In the FAD-binding domain, this increased favourability with respect to ΔH is counter-
balanced by a less favourable entropic measurement. The increased number of potential conformational substates in the full length CPR, because of the presence of the FMN-binding domain, means that a more favourable entropic value is not entirely surprising. Upon nucleotide binding, the presence of the FMN-binding domain has a dual effect; to decrease the $\Delta H$ favourability whilst increasing the $T\Delta S$ favourability. These opposite and almost equal effects are responsible for the similarities in $\Delta G$. The results here appear to corroborate the suggestion that the N-terminal FMN-binding domain affects global energetic properties in full length CPR (Grunau et al., 2006).

These findings strongly suggest that there are differences in the binding strength and energetics of both nucleotide analogues and FMN cofactor between $A. \text{gambiae}$ and human CPRs. Possible structural differences in and around the NADPH binding site may be the cause although as previously mentioned, the events within the FMN-binding domain could also potentially impart an effect. Such differences in the NADPH binding site are an encouraging find when the goal of novel insecticide synthesis in taken into consideration. In order however, to fully expose and utilise such findings may require more comprehensive structural determination.
Chapter 5

5. CATALYTIC AND KINETIC ANALYSIS OF CYTOCHROME P450 REDUCTASE

5.1 Introduction

Elucidation of enzymic activity is a critical aspect in the characterisation of CPR. Since the reductases, expressed without the membrane anchor are unable to interact with a P450, it is not possible to investigate the enzymatic activity of the reductase in the presence of the P450. Instead, the CPR enzymatic activity is studied using non-physiological electron acceptors such as cytochrome c and potassium ferricyanide with the use of these molecules now being a routine and accepted approach. These molecules have proved themselves to be useful diagnostic tools in determining activity and kinetics in these enzymes.
The specific activity of an enzyme is measured by the amount of product formation per milligram of enzyme over a given period of time. It is the measure of the activity of a protein at a specific substrate concentration under a defined set of conditions and hence should be fairly constant for pure enzyme. Measurement of the specific activity can sometimes be useful in determining the purity of a protein sample during the purification process. Cytochrome c binds at the FMN-binding domain and is a measure of the diflavin reductase activity of CPR. Potassium ferricyanide however, binds at FAD-binding domain and is a measure of the C-terminal domain activity, accepting electrons directly from the FAD flavin.

Using the cytochrome c reduction assay, steady state-kinetics can be measured to determine the kinetic parameters $K_m$ and $V_{max}$. $V$ is a measure of the number of reaction catalysed per second per mol of enzyme. The rate of a reaction increases asymptotically with increasing substrate concentration towards the maximal rate, $V_{max}$. With this in mind, it is difficult to define an actual substrate concentration at which the enzyme is fully saturated. Therefore the value of $K_m$ is readily used as an appropriate characterisation of the enzyme as this is the substrate concentration at half the maximal rate; $V_{max}/2$, and is more easily defined.
The specific activity of *A. gambiae* CPR for cytochrome *c* and potassium ferricyanide has been determined. Steady-state kinetics with respect to cytochrome *c* and NADPH were also determined for *A. gambiae* CPR. Human CPR was used throughout as a comparative aid in an attempt to highlight any possible differences in activity and kinetic properties between these two enzymes.
5.2 Methodologies and results

To measure enzyme activity, the rate of change of absorbance of horse heart cytochrome c (Sigma) at 550 nm was measured at 22 °C using a Cary 300 Bio U.V visible spectrophotometer. 1 mL 50 µM cytochrome c (dissolved in either 300 mM potassium phosphate; pH 7.7 or 100 mM Tris-HCl; pH 7.7 containing 1M KCl) was incubated with 14.3 pmol purified CPR. To initiate the reaction, NADPH was added to a final concentration of 50 µM and the change in absorbance measured over a 2 min period. For activity measurements, the extinction coefficient of cytochrome c, 21.41 mM cm\(^{-1}\) was used and each rate measured in triplicate.

CPR is involved in a multi substrate enzyme reaction, with the two substrates being cytochrome c and NADPH. However by keeping one substrate at a constant concentration, the kinetic parameters of the other can be ascertained by experimenting over a large range of concentrations. With this in mind, the kinetic parameters \(K_m\) and \(V_{max}\) were calculated for \(A.\ gambiæ\) and human CPRs with respect to both cytochrome c and NADPH. Once again, buffer influence, in particular that of phosphate, was investigated by performing steady-state kinetics in both phosphate and Tris-HCl based buffers.
To measure ferricyanide reduction, the change in absorbance at 420 nm was measured at 22 °C using a Cary 300 Bio U.V visible spectrophotometer. 1 mL of 1 mM potassium ferricyanide (Sigma) in 50 mM potassium phosphate, pH 7.7 was incubated with purified enzyme (14.3 pmol CPR or 20 pmol AgFAD). The reaction was initiated with NADPH at a final concentration of 50 µM. The change in absorbance was measured over a period of 2 min and the activity measured as µmol ferricyanide reduced per minute per mg enzyme (µmol/min/mg) using the extinction coefficient of reduced potassium ferricyanide of 1.02 mM⁻¹cm⁻¹.

An attempt was made to reconstitute a functional enzyme using the isolated FMN and FAD-binding domains. 1 mg/mL A. gambiae FMN-binding domain and 2.5 mg/mL A. gambiae FAD-binding domain were mixed together in a total volume of 3 mL and left at room temperature with gentle shaking overnight. Samples of the mixture were taken, diluted to 1 mg/mL and used in cytochrome c reduction assays in order to assess activity. A positive result would confirm that the isolated domains could indeed be combined to form a functioning reconstituted enzyme, hence, corroborating the idea that CPR came to be by a genetic fusion event of two separate enzymes, each capable of folding independently (Porter and Kasper, 1986).
5.2.1 Specific activities of cytochrome c and potassium ferricyanide reduction

The specific activities of *A. gambiae* and human CPR’s were measured with respect to cytochrome c reduction. Specific activity was calculated using the measured slope of absorbance change at 550 nm and the extinction coefficient for reduced cytochrome c of 21.41 mM$^{-1}$ cm$^{-1}$. For measurement of the specific activity of potassium ferricyanide reduction, the negative change in absorbance at 420 nm was measured and the extinction coefficient of reduced potassium ferricyanide; 1.02 mM$^{-1}$ cm$^{-1}$. An example of a typical slope of a potassium ferricyanide reduction is shown (Figure 5.2).
Figure 5.1. A typical cytochrome c reduction curve. The change in absorbance at 550 nm is measured over a period of time, in this case 60 s. In this example, 50 µM cytochrome c was made up in 100 mM Tris-HCl, pH 7.7 containing 100 mM KCl. 14.3 pmol human CPR was added and the reaction started by addition of NADPH to a final concentration of 50 µM. The absorbance was measured at 22 °C using a Cary 300 Bio U.V visible spectrophotometer. A control experiment which contained no enzyme was performed to confirm that there was no background absorbance change in enzyme deficient reactions.
Figure 5.2. A typical potassium ferricyanide reduction curve. The change in absorbance at 420 nm is measured over a period of time, in this case 120 s. In this example, 1 mM potassium ferricyanide was made up in 50 mM phosphate, pH 7.7. 20 pmol *A. gambiae* FAD-binding domain was added and the reaction started by addition of NADPH to a final concentration of 50 µM. The absorbance was measured at 22 °C using a Cary 300 Bio U.V visible spectrophotometer. A control experiment which contained no enzyme was performed to confirm that there was no background absorbance change in enzyme deficient reactions.
5.2.1.1 Cytochrome c reduction

The specific activity, with respect to cytochrome c reduction, when measured in Tris-HCl based buffer was 14.08 µmol min\(^{-1}\)mg\(^{-1}\) for *A. gambiae* CPR and 11.28 µmol min\(^{-1}\)mg\(^{-1}\) for human CPR. These values are similar, with *A. gambiae* CPR appearing to have an ever so slightly higher activity for cytochrome c reduction.

Traditionally, cytochrome c assays are performed in 300 mM phosphate buffer. However, the known inhibitory effect of free phosphate on NADPH binding could potentially diminish activity in this buffer. Phosphate has been shown to lower the binding affinity of nicotinamide nucleotides to CPR (Grunau *et al*., 2006). Tris-HCl buffer does not cause the problem of free phosphate affecting coenzyme binding, however, 100 mM Tris-HCl is not an ideal buffer from a physiological perspective. Phosphate is a much more favourable buffer base which highlights an interesting dilemma regarding the choice of buffer for specific activity measurement. To address this issue, the same assay was also performed in 300 mM phosphate buffer to determine the buffer best suited to cytochrome c reduction by CPR: the less physiologically relevant Tris-HCl or the more favourable, yet potentially prone to inhibitory effects, phosphate buffer. The specific activity of cytochrome c reduction in phosphate buffer by *A. gambiae*
CPR was calculated to be 23.85 µmol min$^{-1}$mg$^{-1}$ whilst the specific activity for human CPR was 18.27 µmol min$^{-1}$mg$^{-1}$.

The observation is similar in phosphate buffer in that *A. gambiae* has slightly higher specific activity than human CPR. There is a difference however in specific activities in the two buffers. There is between a 1.5 – 2 fold increase in activity in both *A. gambiae* and human CPR when the assay was performed in phosphate buffer. This shows that despite the potential inhibitory effects, phosphate is a much more favourable buffer for cytochrome *c* reduction. Despite some inhibitory effects of the phosphate buffer, its physiological relevance tends to make this a preferred buffer rather than the Tris-HCl buffer.

It has been shown that *A. gambiae* CPR is FMN, and to a lesser extent FAD, deficient relative to the human enzyme. Work on *A. minimus* CPR, which is also binds both flavins weakly, concluded that the addition of exogenous FAD during cytochrome *c* and potassium ferricyanide reduction improved specific activity by up to 2-fold. To determine if this was the case in *A. gambiae* CPR, the specific activity of cytochrome *c* was measured following the addition of a 10-fold excess of FMN to the protein sample. The specific activity of *A. gambiae* CPR with exogenous FMN was almost identical to that which was calculated without additional flavin. The same was seen when exogenous FAD was added to the reaction suggesting that *A. gambiae*
CPR is fully saturated with flavin. By way of comparison, the specific activity of cytochrome c reduction by human CPR was also investigated following exogenous flavin addition. Again, as for the *A. gambiae* CPR, there was no improvement of the specific activity with a 10-fold excess of FMN and FAD.

The specific activities of measured CPRs have been shown to lie within the range of 30 -60 µmol min⁻¹ mg⁻¹ (Shen *et al.*, 1989; Murataliev *et al.*, 1999) At specific activities of, respectively, 23.85 and 18.27 µmol min⁻¹ mg⁻¹ in phosphate buffer for both *A. gambiae* and human CPR, these specific activities seemed a little low. One potential reason for this could be the temperature at which the reactions were performed. Cytochrome c reduction assays are traditionally performed between 25 and 30 °C, however, due to the lack of a thermostat on the spectrophotometer used; these data were collected at room temperature which was readily maintained at 22 °C. The lower temperature used for these assays could account for the low specific activities and in order to investigate this further, an alternative spectrophotometer; one with a functioning thermostat, was trialled. The same samples had their specific activities measured at 25 °C and the increased temperature had an effect on specific activity. There was an approximate 2-fold increase in specific activity when measured at 25 °C; with 42.21 and 35.07 µmol min⁻¹ mg⁻¹ calculated for *A. gambiae*
and human CPR respectively. For the human protein, this value agrees with published values (Smith et al., 1994). The specific activities of \textit{A. gambiæ} and human CPR’s under all conditions are summarised in Table 5.1.
Table 5.1. Cytochrome c reduction data of *A. gambiae* and human CPR under a variety of conditions. The buffers used were either 100 mM Tris-HCl; pH 7.7 containing 100 mM KCl or 300 mM phosphate buffer; pH 7.7. In each case, 50 µM cytochrome c was reduced using 14.3 pmol CPR. The reactions were initiated by addition of NADPH to a final concentration of 50 µM. The absorbance was measured at either 22 °C or 25 °C using a Cary 300 Bio U.V visible spectrophotometer. Using the extinction coefficient of 21.41 mM⁻¹ cm⁻¹ for reduced cytochrome c, the slope of change in absorbance was converted to specific activity. The quoted errors are standard errors derived from triplicate reactions.

<table>
<thead>
<tr>
<th>Specific activity (µmol/mg/min)</th>
<th>Tris Buffer</th>
<th>Phosphate Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. gambiae CPR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 °C</td>
<td>14.08 ± 0.52</td>
<td>23.85 ± 1.10</td>
</tr>
<tr>
<td>25 °C</td>
<td>-</td>
<td>42.21 ± 3.96</td>
</tr>
<tr>
<td><strong>Human CPR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 °C</td>
<td>11.28 ± 0.37</td>
<td>18.27 ± 0.96</td>
</tr>
<tr>
<td>25 °C</td>
<td>-</td>
<td>35.07 ± 1.42</td>
</tr>
<tr>
<td><strong>Regenerated CPR</strong></td>
<td>-</td>
<td>0.78 ± 0.12</td>
</tr>
</tbody>
</table>
5.2.1.2 Potassium ferricyanide reduction

The specific activity for potassium ferricyanide reduction of *A. gambiae* CPR was 16.28 µmol min$^{-1}$ mg$^{-1}$ whilst the specific activity of human CPR which was calculated to be 12.41 µmol min$^{-1}$ mg$^{-1}$. Although these values are similar, the specific activity of the *A. gambiae* CPR is slightly higher than that of the human CPR, indicating a more active FAD-binding domain in *Anopheline* CPR compared to the human equivalent. Once again; these measurements were taken at 22 °C and it is possible that raising the temperature up to 25-30 °C could increase these specific activities further by maximising the reaction conditions.

Potassium ferricyanide reduction is a measure of the activity of the FAD-binding domain as it accepts electrons directly from the FAD cofactor. Since the binding of the coenzyme, NADPH, and the substrate, potassium ferricyanide, are both contained within the single FAD-binding domain, the activity of the isolated FAD-binding domain can also be assessed using this assay. The specific activity of the isolated *A. gambiae* FAD-binding domain was 28.31 µmol min$^{-1}$ mg$^{-1}$; approximately 1.5 - 2-fold higher than the calculated specific activity of the full length *A. gambiae* CPR (Table 5.2). The increased activity of the isolated FAD-binding domain relative to the full length enzyme
could prove to be significant when considering the overall activity of
*A. gambiae* CPR.

5.2.1.3 Regeneration of CPR using the isolated flavin-binding domains

The isolated FMN and FAD-binding domains are homologous to
flavodoxin and FNR respectively and are thought to attribute their
ancestry to these domains (Porter and Kasper, 1986). This is most
likely the reason why the isolated binding domains are able to be
expressed and fold independently of each other. It is therefore feasible
that the FMN and FAD-binding domains can be combined to
regenerate an active CPR enzyme. The specific activity for cytochrome
*c* reduction of *A. gambiae* CPR was 23.85 μmol min⁻¹ mg⁻¹ in
phosphate buffer however the specific activity of the regenerated
enzyme dropped to 0.78 μmol min⁻¹ mg⁻¹: only 3.3 % of the activity of
the full length enzyme (Table 5.3). The lack of activity of the
reconstituted CPR is illustrated by comparing the observed slopes of
this regenerated enzyme to the intact reductase (Figure 5.3)
Table 5.2. Potassium ferricyanide reduction of *A. gambiae* CPR, human CPR and *A. gambiae* isolated FAD-binding domain. The buffer used was 50 mM phosphate buffer; pH 7.7. In each case, 1mM potassium ferricyanide was reduced using 14.3 pmol CPR or 20 pmol FAD binding domain. The reactions were initiated by addition of NADPH to a final concentration of 50 μM. The absorbance was measured at either 22 °C using a Cary 300 Bio U.V visible spectrophotometer. Using the extinction coefficient of 1.02 mM⁻¹ cm⁻¹ for reduced potassium ferricyanide, the slope of change in absorbance was converted to specific activity. The quoted errors are standard errors derived from triplicate reactions.

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (μmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human CPR</strong></td>
<td>12.41 ± 0.71</td>
</tr>
<tr>
<td><strong>A. gambiae CPR</strong></td>
<td>16.28 ± 1.36</td>
</tr>
<tr>
<td><strong>A. gambiae FAD-binding domain</strong></td>
<td>28.31 ± 1.12</td>
</tr>
</tbody>
</table>
Figure 5.3. The relative cytochrome c reduction capabilities of *A. gambiae* CPR and the regenerated FAD/FMN-binding domain reductase. The reactions took place in 300 mM phosphate; pH 7.7 at 22 °C. In each case the reaction was initiated by addition of NADPH to a final concentration of 50 µM. A = 14.3 pmol *A. gambiae* CPR B = Equimolar concentrations of isolated FMN and FAD-binding domains to a final concentration of 1.43 µM. The absorbance was measured using a Cary 300 Bio U.V visible spectrophotometer.
This confirms that although a functioning reductase can be regenerated from the two flavin binding domains, its activity is poor. This is due to the lack of the inter-domain linker region. This unstructured domain shows a high degree of flexibility and is responsible for bringing the FMN and FAD flavins in close proximity to each other, and for orientating the domains in order for efficient electron transfer to occur. In the regenerated enzyme, although both flavins are present, the lack of the linker regions makes inter-flavin electron transfer vastly more difficult, hence the poor specific activity relative to the fully intact enzyme.

5.2.2 CPR steady-state kinetics

Reduction by CPR involves interaction with two substrates; NADPH and the terminal acceptor. Multi-substrate enzyme reactions often follow complex rate equations to assess the binding of the various substrates making data analysis difficult. This can be simplified if the concentration of one substrate is kept constant whilst the other is varied over a wide range of concentrations. Under these conditions, the enzyme behaves like a single-substrate enzyme and if \( v \) vs \([S]\) is plotted, the \( K_m \) and \( V_{max} \) of the varied substrate can be calculated. The cytochrome \( c \) reduction assay was used in order to ascertain kinetic
parameters $K_m$ and $V_{\text{max}}$ with respect to NADPH and cytochrome $c$ itself. Due to the proposed random bi-bi kinetic mechanism observed in CPR, the use of the term $K_m$ is somewhat misleading. In this scheme the $K_m$ values for both NADPH and cytochrome $c$ will likely be interchangeable and be altered as the concentration of each substrate changes. Since the $K_m$ values for each substrate were measured at a single fixed concentration of the other, all $K_m$ values reported are not true $K_m$ and should be taken to mean apparent $K_m$.

For determination of cytochrome $c$ kinetics, a series of cytochrome $c$ concentrations were made (0, 1, 2, 4, 5, 10, 15, 20, 25, 50, 75, 100 µM). 10 µL 0.1 mg/mL purified enzyme was incubated with 1 mL of each cytochrome $c$ concentration. Again, the reaction was initiated by addition of NADPH to a final concentration of 50 µM and the change in absorbance measured over 2 min. For kinetic analyses, each reaction was performed in duplicate. For determination of NADPH kinetics, 10 µL 14.3 pmol purified enzyme was incubated with 1 mL 50 µM cytochrome $c$. The reactions were initiated by the addition range of NADPH concentrations (0, 1, 2, 4, 5, 10, 15, 20, 25, 50, 75, 100, 150, µM final concentrations) and the change in absorbance measured over 2 min. Again, for NADPH kinetic analyses, the reactions were performed in duplicate.
Having observed a difference in specific activity between Tris-HCl and phosphate buffers, kinetic parameters were determined for the reactions performed in both buffers to check that this difference transfers to kinetic parameters as well as specific activity. Reaction temperature has been shown to have an effect on specific activity however experiments performed at 22 °C still show a consistent reasonable rate of reaction. Because of this, all kinetic experiments were run at 22 °C. It was also shown that the addition of exogenous flavins prior to the reaction showed had little effect thus they were not added before the commencement of kinetic experiments.

5.2.2.1 Phosphate buffer kinetics

Following the series of experiments using a range of cytochrome c concentrations, the $K_m$ and $V_{max}$ values were calculated. In phosphate buffer, the buffer more suited to enzyme activity, the $K_m$ of *A. gambiae* CPR for cytochrome c was 24.21 µM. This value was similar, albeit slightly higher than the human CPR value which was 18.01 µM. There is a similar observation when the $K_m$ values for NADPH are considered. In *A. gambiae* CPR, the $K_m$ for NADPH was calculated to be 26.03 µM whilst in human CPR the $K_m$ dropped to 15.04 µM (Table 5.3). Both *A. gambiae* and human CPR fit to the Michaelis-Menten
Figure 5.4. Steady state kinetic data of *A. gambiae* and human CPR in phosphate based buffer. 

**A** = Kinetics with respect to cytochrome c. A range of cytochrome c concentrations, 0-100 µM, were tested. The concentration of cytochrome c was plotted against the rate of reaction when the concentration of NADPH was kept at a constant 50 µM. 

**B** = Kinetics with respect to NADPH. A range of NADPH concentrations, 0-150 µM, were tested. The concentration of NADPH was plotted against the rate of reaction when the concentration of cytochrome c was kept at a constant 50 µM. The reactions took place in 300 mM phosphate; pH 7.7 at 22 °C. Red line = Human CPR; Blue line = *A. gambiae* CPR.
Table 5.3. Apparent $K_m$ values of *A. gambiae* and human CPR with respect to cytochrome c and NADPH in phosphate buffer. The apparent $K_m$ values were calculated using SigmaPlot v.11 using data from Figure 5.4. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cytochrome c</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gambiae</em> CPR</td>
<td>24.21 ± 4.05</td>
<td>26.03 ± 4.40</td>
</tr>
<tr>
<td>Human CPR</td>
<td>18.01 ± 3.15</td>
<td>15.04 ± 3.03</td>
</tr>
</tbody>
</table>
kinetic model (Figure 5.4). These data indicate that the mode of substrate binding is similar in both *A. gambiae* and human CPR although the human CPR shows stronger interaction with each substrate than *A. gambiae* CPR.

The $V_{\text{max}}$ of human and *A. gambiae* CPR was calculated with respect to each of the substrates. $V_{\text{max}}$ is the maximal rate of reaction and is expressed as the amount of substrate reduced per minute per amount of CPR (nmol/min/nmol). The $V_{\text{max}}$ of *A. gambiae* CPR with respect to cytochrome c was 1562 nmol/min/nmol which was similar to that calculated for human CPR of 1488 nmol/min/nmol. The $V_{\text{max}}$ of the same enzymes with respect to NADPH were 1466 nmol/min/nmol and 1568 nmol/min/nmol for *A. gambiae* and human CPR respectively (Table 5.4). These values are again similar however the human CPR shows a slightly higher $V_{\text{max}}$ with respect to NADPH than *A. gambiae* CPR.

Due to the deficiency in FMN and FAD observed in *A. gambiae* CPR when compared to human CPR, a truer indication of enzyme activity would be to express $V_{\text{max}}$ as the amount of substrate reduced per minute per the amount of flavin cofactor (nmol/min/nmol flavin). This allows the lack of bound flavin to be taken into account when considering the rate of reaction. When the flavin content is taken into account, there is a slight change in the pattern of activity. The $V_{\text{max}}$ values with respect
to cytochrome c in *A. gambiae* CPR were 1951 and 2171 nmol/min/nmol FAD and FMN respectively compared to 1623 and 1686 nmol/min/nmol FAD and FMN respectively in human CPR.

The $V_{\text{max}}$ values which had been very similar when expressed with respect to enzyme concentration become increased in *A. gambiae* when flavin content is considered. The $V_{\text{max}}$ with respect to NADPH show a reversal when expressed as a function of flavin concentration. The values are 1832 and 2038 nmol/min/nmol FAD and FMN respectively in *A. gambiae* CPR which and 1711 and 1776 nmol/min/nmol FAD and FMN respectively in human CPR (Table 5.5). These values are higher in *A. gambiae* CPR when the reverse was true when $V_{\text{max}}$ was expressed relative to enzyme concentration.

These data highlight how the rate of reaction value can be misleading depending on how the units are expressed. CPR activity is dependent on reaction conditions and substrate concentrations but it is also heavily influenced by the FMN and FAD concentrations. Thus for direct comparison of rates, due to the obvious flavin deficiency in *A. gambiae* CPR, expressing $V_{\text{max}}$ as a function of flavin concentration is a more appropriate measure of the rate.
Table 5.4. \( V_{\text{max}} \) values of \( A. \ gambiae \) and human CPR with respect to cytochrome \( c \) and NADPH in phosphate-based buffer. The \( V_{\text{max}} \) values were calculated using SigmaPlot v.11 using data from Figure 5.4. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cytochrome ( c )</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A. \ gambiae ) CPR</td>
<td>1562 ± 90</td>
<td>1466 ± 87</td>
</tr>
<tr>
<td>Human CPR</td>
<td>1488 ± 88</td>
<td>1568 ± 75</td>
</tr>
</tbody>
</table>

Table 5.5. \( V_{\text{max}} \) values of \( A. \ gambiae \) and human CPR with respect to cytochrome \( c \) and NADPH in phosphate based buffer expressed as a function of flavin concentration. The \( V_{\text{max}} \) values are the same as in table 5.4 however expressed as a function of flavin concentration rather than reductase concentration. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th>`Enzyme</th>
<th>Cytochrome ( c )</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAD</td>
<td>FMN</td>
</tr>
<tr>
<td>( A. \ gambiae ) CPR</td>
<td>1951 ± 112</td>
<td>2171 ± 125</td>
</tr>
<tr>
<td>Human CPR</td>
<td>1623 ± 96</td>
<td>1686 ± 99</td>
</tr>
</tbody>
</table>
5.2.2.2 Tris-HCl buffer kinetics

The steady-state kinetic experiments were repeated in Tris-HCl buffer in order to assess any potential influence of the buffer on $K_m$ and $V_{max}$. With respect to cytochrome $c$, the $K_m$ of $A. gambiae$ CPR is 12.68 µM which is similar to the 8.70 µM measured for human CPR. There is more of a difference seen in the $K_m$ values with respect to NADPH. For $A. gambiae$ CPR, a value of 9.24 µM was measured which is significantly higher than the human CPR value of 5.70 µM (Table 5.6).

Again, both $A. gambiae$ and human CPR data fit to the Michaelis-Menten model and both enzymes adhere to a similar mode of substrate binding (Figure 5.5).

The clearest observation is in the comparison of Tris-HCl and phosphate buffer $K_m$ values. There is an approximate 3-fold decrease in the $K_m$ values of both enzymes with respect to NADPH in Tris-HCl. This is not unexpected, especially when the known inhibitory effect of free phosphate is taken into consideration. There is also a decrease in the $K_m$ values with respect to cytochrome $c$ in Tris-HCl albeit less of a decrease than what was observed with respect to NADPH. The $V_{max}$ of $A. gambiae$ with respect to cytochrome $c$ was 1000 nmol/min/nmol compared to that of human CPR which was 1125 nmol/min/nmol.
Figure 5.5. Steady state kinetic data of *A. gambiae* and human CPR in Tris-HCl based buffer. **A** = Kinetics with respect to cytochrome c. A range of cytochrome c concentrations, 0-100 µM, were tested. The concentration of cytochrome c was plotted against the rate of reaction when the concentration of NADPH was kept at a constant 50 µM. **B** = Kinetics with respect to NADPH. A range of NADPH concentrations, 0-150 µM, were tested. The concentration of NADPH was plotted against the rate of reaction when the concentration of cytochrome c was kept at a constant 50 µM. The reactions took place in 100 mM Tris-HCl; pH 7.7 containing 100 mM KCl at 22 °C. Red line = Human CPR; Blue line = *A. gambiae* CPR.
Table 5.6. Apparent $K_m$ values of *A. gambiae* and human CPR with respect to cytochrome $c$ and NADPH in Tris-HCl based-buffer. The apparent $K_m$ values were calculated using SigmaPlot 11 using data from Figure 5.5. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cytochrome $c$</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gambiae</em> CPR</td>
<td>12.68 ± 2.49</td>
<td>9.24 ± 1.17</td>
</tr>
<tr>
<td>Human CPR</td>
<td>8.70 ± 1.54</td>
<td>5.70 ± 0.67</td>
</tr>
</tbody>
</table>
Table 5.7. $V_{\text{max}}$ values of *A. gambiae* and human CPR with respect to cytochrome c and NADPH in Tris-HCl-based buffer. The $V_{\text{max}}$ values were calculated using SigmaPlot v.11 using data from Figure 5.5. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome c</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. gambiae CPR</strong></td>
<td>1000 ± 84</td>
<td>858 ± 46</td>
</tr>
<tr>
<td><strong>Human CPR</strong></td>
<td>1125 ± 53</td>
<td>1156 ± 23</td>
</tr>
</tbody>
</table>

Table 5.8. $V_{\text{max}}$ values of *A. gambiae* and human CPR with respect to cytochrome c and NADPH in Tris-HCL based buffer expressed as a function of flavin concentration. The $V_{\text{max}}$ values are the same as in table 5.7 however expressed as a function of flavin concentration rather than reductase concentration. The quoted errors are standard errors derived from duplicate reactions.

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome c</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAD</td>
<td>FMN</td>
</tr>
<tr>
<td><strong>A. gambiae CPR</strong></td>
<td>1250 ± 105</td>
<td>1391 ± 117</td>
</tr>
<tr>
<td><strong>Human CPR</strong></td>
<td>1227 ± 57</td>
<td>1274 ± 60</td>
</tr>
</tbody>
</table>
These are very similar not only to each other, but also to the $V_{\text{max}}$ values with respect to NADPH. For *A. gambiae*, this was 858 nmol/min/nmol and for human CPR, 1156 nmol/min/nmol (Table 5.7). As described previously, a more appropriate unit for $V_{\text{max}}$ takes into account the flavin concentration. When this was applied, for *A. gambiae* CPR, the $V_{\text{max}}$ with respect to cytochrome $c$ were 1250 and 1391 nmol/min/nmol FAD and FMN respectively compared to 1072 and 1193 nmol/min/nmol FAD and FMN respectively with respect to NADPH. In general, these are similar to what was observed for human CPR. With respect to cytochrome $c$, the $V_{\text{max}}$ were 1227 and 1274 nmol/min/nmol FAD and FMN respectively whilst with respect to NADPH, the human CPR $V_{\text{max}}$ were 1261 and 1309 nmol/min/nmol FAD and FMN respectively (Table 5.8).

These data are very similar to what was observed when the reactions were performed in phosphate buffer. One difference is that the $V_{\text{max}}$ with respect to NADPH in *A. gambiae* CPR is lower than human CPR in Tris-HCl buffer whilst it is higher than human CPR in phosphate; any differences in $V_{\text{max}}$ observed between *A. gambiae* and human CPR are negligible suggesting that, in general, the rates of reaction in these enzymes are very similar. The largest difference when the $V_{\text{max}}$ data are compared is that the values measured in phosphate buffer are 1.5 – 2-fold higher than those from the Tris-HCl buffer. All in all, the type of
buffer used in cytochrome c reaction does have an influence on the kinetic properties of A. gambiae and human CPR’s without affecting the overall mode of substrate binding of these enzymes.
5.3 Discussion

The specific activities and steady-state kinetic parameters of both *A. gambiae* and human CPR’s were measured. The data for the two enzymes were directly compared to each other. However, for an additional level of comparison to investigate the effects of two buffers, Tris-HCl vs phosphate was also undertaken. The inhibitory effect of free phosphate on NADPH interaction with CPR is well known (Murataliev *et al.*, 1999) and the possibility of this inhibition affecting both activity and kinetic parameters was the rationale for the additional study in phosphate-free buffer.

The specific activities were measured with cytochrome *c*; to assess the diflavin reductase activities of these enzymes whilst potassium ferricyanide was used to measure to efficiency of the FAD-binding domain. In general, the activities of *A. gambiae* and human CPR with respect to both electron acceptors were very similar with *A. gambiae* CPR being slightly more active in both cases. The largest differences were seen when the two buffer types were compared for cytochrome *c* reduction. Both enzymes were approximately 1.5 – 2-fold more active in phosphate buffer compared to Tris-HCl. Why this should be the case is not entirely clear. Since phosphate is a more physiological buffer, it follows that the data from experiments performed in this buffer is more relevant.
The increased specific activity observed in phosphate buffer is corroborated by the $V_{\text{max}}$ values. These are also increased with respect to both cytochrome $c$ and NADPH for both the $A. \ gambiae$ and human enzymes confirming that phosphate is the preferable buffer for investigating CPR activity. The $K_m$ values measured in these two buffers support the theory surrounding the inhibitory effect of free phosphate with regards to NADPH binding. For both enzymes, there was an approximate 3-fold increase in $K_m$ in phosphate buffer conditions. This clearly suggests an inhibitory effect of phosphate derived buffer on CPR affinity for NADPH.

The potassium ferricyanide reduction assay allowed the isolated FAD-binding domain of $A. \ gambiae$ CPR to be studied alongside the full length domain. These data show that the isolated domain was much more active, approximately 1.5 – 2-fold, than the full length protein. It is possible that the FMN-binding domain in the full length enzyme has an ‘inhibitory’ effect on potassium ferricyanide reduction by the FAD-binding domain. This could be through an overall conformation or spatial constraints.

$A. \ minimus$ CPR, another mosquito reductase, was shown to have a deficiency in FMN and FAD cofactors (Sarapusit et al., 2008) and the addition of exogenous flavins prior to cytochrome $c$ reduction increases the specific activity of this enzyme. The binding of the
cofactors FMN and FAD to A. gambiae CPR is relatively weak and recombinant A. gambiae CPR is deficient in both cofactors. Unlike A. minimus CPR, the addition of exogenous FMN and FAD just prior to the enzyme reaction had little to no effect on the specific activity of this enzyme. This suggests that despite the low level of the diflavin cofactors, particularly FMN, the enzyme appears to be fully active.

A further study involved the regeneration of an active reductase from the constituent FMN and FAD-binding domains. These were recombined to form an active reductase. However the actual activity was only 3 - 4 % of that of the intact enzyme. This is due to the lack of the inter-domain hinge region. It is absolutely clear that this region is entirely responsible for bringing the flavin cofactors into a proximity which encourages efficient electron transfer.

The specific activity and kinetic data do not themselves fully explain the action of CPR, be it in A. gambiae or human. The main aspect to be gained from this is that the buffer conditions are crucial and the free-phosphate inhibitory effect has been confirmed. There are aspects of the data which can be easily explained such as the specific activity and $K_m$ values in phosphate and phosphate-free buffers. Some aspects however; such as the similarities in specific activities and $V_{max}$ values of A. gambiae and human CPR and how this relates to the anomalous observed FMN-binding, are a lot more difficult to explain using these
data alone. It is therefore important to realise that in order to fully understand and interpret kinetic data, a greater level of in-depth structural information is an essential requirement.
Chapter 6

6. GENERAL DISCUSSION

The main aim of this work was the expression of recombinant *A. gambiae* cytochrome P450 reductase with a view to biophysical characterisation by NMR. A considerable amount of CPR research has focussed on the mechanisms of kinetics and electron transfer (Murataliev *et al.*, 2004) whilst aside from a few notable exceptions (Wang *et al.*, 1997; Lamb *et al.*, 2006), the structural nuances are less well understood. Malaria is a significant disease with *A. gambiae* being the principal vector in the Sub-Saharan African region.

Before this work was undertaken, very little was known about *A. gambiae* CPR except the intriguing finding that CPR knocked-out strains were permethrin susceptible (Lycett *et al.*, 2006). Preliminary work also highlighted that the use of a standard 2’, 5’-ADP sepharose during recombinant *A. gambiae* CPR purification was consistently unsuccessful due to poor interaction with the resin and was
corroborated by inhibition studies (unpublished data). Over recent years, the efficacy of modern day insecticides has been on the wane due to growing populations of resistant mosquito strains. Moreover, monooxygenase-mediated in common and often involving the up-regulation of cytochromes P450 (Vulule et al., 1994), and in this mechanism, CPR interaction with cytochromes P450 is pivotal. The impact of *A. gambiae* CPR in relation to permethrin susceptibility allied to the proposed weak interaction with 2’’, 5’-ADP has made *A. gambiae* CPR a tempting and exciting target for a potential novel class of insecticides for malarial control (Lycett et al., 2006).

Much is known about a number of mammalian CPRs, namely rat and human in addition to the insect CPRs, house fly and *A. minimus*. Before this work, *A. gambiae* CPR had not been studied in any detail, thus, through enzyme characterisation, its behaviour could be analysed both in isolation as well as relative to that of the more established CPRs. Throughout the course of this thesis, the human CPR has been used primarily as a control enzyme for direct comparison to *A. gambiae* data. Human CPR has been reasonably well studied and shows incredible sequence homology to rat CPR, which provided the first CPR structure (Wang et al., 1997) with 96 % similarity (Figure 1.10). The main rationale in including human CPR in this study impinges on *A. gambiae* CPR as a potential insecticide target. For this
to be the case, any novel compounds need to specifically target *A. gambiae* CPR, thus, any possible action against or effects on the human monooxygenase system need to be fully realised and assessed.

For any characterisation, be it biochemical or biophysical, to be carried out, the recombinant protein must be efficiently expressed in a folded, active form meaning an effective expression system needs to be established. Throughout this work, BL-21 Codon Plus RP competent *E. coli* were utilised for recombinant protein expression and the system optimised for the production of reasonably high yields. 1D proton NMR experiments were performed to determine whether the enzyme was fully folded and for *A. gambiae* samples, this was the case. Activity assays using non-physiological electron acceptors cytochrome *c* and potassium ferricyanide, which assess electron transfer capabilities from the FMN-binding and FAD-binding domain respectively, were used to confirm that this correctly folded enzyme was indeed active (chapter 5).

CPR consists of two principal domains, the FMN and FAD-binding domains, connected by an unstructured linker region (Figure 1.10). The homology of these FMN and FAD-binding domains to the bacterial enzymes flavodoxin and FNR respectively (Porter and Kasper, 1986) strongly suggests that the individual components could be studied as separate and independent entities. This was achieved and the isolated
flavin binding domains were assessed for cofactor binding, enzyme activity and biophysical characterisation.

Possibly the most significant role of the isolated domains was in the determination of redox potentials (section 3.2.5). In this case, the isolated flavins are analysed to corroborate the data generated for the intact enzyme and ease the convolution of redox potential data. Although in this thesis, only the FMN-binding domain had its data fully deconvoluted, experiments involving the FAD-binding domain and intact CPR were carried out. A combination of sample behaviour and time constraints prevented full data analysis from being carried out. Despite the only calculated redox potentials being of the FMN-binding domain, when compared to the human equivalent (Munro et al., 2001) they are still of interest. The oxidised/semiquinone transition in human FMN-binding domain was -43 mV which is much more positive than the -92 mV seen in A. gambiae FMN-binding domain (Table 3.2). This suggests that in the oxidised form, human FMN accepts electrons more readily than the Anopheline equivalent which is possibly due to a weaker binding of FMN to its binding site.

Differences in the binding affinity of FMN to its binding site between human and A. gambiae CPR was an interesting, yet not completely unexpected, discovery. Mosquito CPR, derived from A. minimus, has been shown to be deficient in both FMN and FAD cofactors (Sarapusit

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et al., 2008) and a similar trait could be seen in *A. gambiae* CPR. In fact, when the flavin content of *A. gambiae* CPR was analysed, there was only a 0.72:1 FMN: protein ratio which represents a 20% deficiency in FMN relative to human CPR (Table 3.1). The specific activity, calculated as a function of cytochrome c reduction, of *A. gambiae* CPR was not improved following the addition of exogenous FMN of FAD to the reaction mixture. This implies that FMN deficient *A. gambiae* CPR is still fully active suggesting this enzyme contains a naturally low level of bound flavin cofactors, particularly FMN.

The data regarding the activity of *A. gambiae* CPR relative to the concentration of bound flavin is somewhat anomalous as it would be expected that in an enzyme deficient in FMN and FAD, the addition of exogenous flavin would increase enzyme activity – as was shown with *A. minimus* (Sarapusit et al., 2008). As this is not the case for *A. gambiae* CPR, it is likely that there are subtle structural influences that affect flavin binding and enzyme activity which have yet to be resolved meaning the observations presented in this thesis remain unexplained.

Further evidence for a difference in FMN interaction between *A. gambiae* and human CPRs was highlighted using Isothermal Titration Calorimetry (ITC) (section 4.2.4). Using the ITC\textsubscript{200} system (MicroCal), FMN was sequentially titrated into the apo-form of the FMN-binding
domain of both *A. gambiae* and human CPR. The resultant binding isotherms suggested an approximate 4-fold decreased affinity for FMN in *A. gambiae* FMN-binding domain compared to the human domain (Table 4.4). Ideally this would have been corroborated using FAD bound, FMN apo-CPR samples to determine whether this behaviour was replicated in the full length CPR. The FAD-binding site would have to be fully saturated in order to prevent FMN binding to the FAD-binding domain and clouding any data. Unfortunately, the instability of the apo-form of both *A. gambiae* and human CPR prevented such experiments being carried out. The same was also observed for the FAD-binding domains indicating that this domain is the driving force behind apo-CPR instability.

Throughout this work, ITC was primarily used to probe the NADPH binding site in order to establish possible differences in coenzyme nucleotide binding properties between the *A. gambiae* and human CPRs (chapter 4). The finding that 2',5'-ADP binds up to 10-fold less strongly to *A. gambiae* CPR than to the human CPR was intriguing and suggested a difference in coenzyme binding which could potentially impinge on basal activity of the mosquito reductase. The weaker affinity of *A. gambiae* CPR for 2', 5'-ADP was confirmed by ITC (section 4.2.2) and analysis of further nucleotide analogues was probed.
The 2’-phosphate moiety of NADPH was shown to be essential for interaction of the coenzyme to CPR. NADP\(^+\) bound much more strongly to both A. gambiae and human CPRs than NAD\(^+\) which was simply down to the presence of an additional phosphate group at the 2’ position in NADP\(^+\). This 2’-phosphate group is also responsible for the majority of the binding energy in NADPH judging by the similarities between NADP\(^+\) and 2’, 5’-ADP binding affinities and thermodynamics. The fact that these values, observed in both A. gambiae and human CPRs, were so similar suggested that the incorporation of the nicotinamide moiety, present in NADP\(^+\), had little impact on the binding characteristics of the ligands. This finding corroborates previous findings (Grunau et al., 2006) and confirms that A. gambiae CPR adheres to known conventions regarding the bipartite nature of NADPH binding in these enzymes (Deng et al., 1999).

Comparison of 2’, 5’-ADP and 2’-AMP binding data also shows that although the significant interaction is at the 2’-phosphate, addition of the 5’-phosphate group improves the binding affinity by at least an order of magnitude. This is likely due to secondary interactions and has previously been observed in human CPR (Grunau et al., 2006).

The increased binding affinity of this range of nucleotide analogues for human CPR was confirmed by the thermodynamic parameters (section 4.2.3). A very interesting observation was that in A. gambiae CPR, the
isolated FAD-binding domain bound these ligands quite differently than the intact CPR (Tables 4.2 and 4.3). Although the overall binding energies ($\Delta G$) were apparently the same, the values for $\Delta H$ and $T\Delta S$ suggest key differences in binding. It is possible that the presence of the FMN-binding domain in the intact enzyme could be at least partly responsible for these differences in thermodynamic characteristics. The very fact that upon NADPH binding, FMN is brought close to the FAD-binding domain to allow swift, efficient electron transfer is potentially energetically detrimental in the intact enzyme. In the FAD-binding domain binding, where no FMN-binding domain is present, there is a more favourable observed $\Delta H$ value than the intact enzyme. It is quite possible that some of the binding energy is expended to necessitate the conformational shift of the FMN-binding domain in the intact enzyme.

Despite both deficiency in flavin cofactors and apparent weaker NADPH interaction, the activity of cytochrome c and potassium ferricyanide reduction of A. gambiae CPR is not too dissimilar to human CPR (section 5.2.1). In fact, the A. gambiae CPR was more active for reduction of these electron acceptors in laboratory conditions. Although there was little observed difference in enzyme activity between A. gambiae and human CPR, the calculated kinetic parameters $K_m$ and $V_{max}$ indicated a possible contrast in how these
activities are achieved. The lower $K_m$ values measured with respect to both NADPH and cytochrome $c$ in human CPR suggested a stronger affinity for both these substrates in the human enzyme (Table 5.3). When the ITC nucleotide binding study (chapter 4) is considered, in the case of NADPH, this is not surprising but this apparent improved affinity for cytochrome $c$ in human CPR is potentially more interesting. It has been suggested that the cytochrome $c$ and cytochrome P450 binding sites in CPR are either very close or indeed overlap (Tamburini and Schemkman, 1986). Therefore, a possible weaker affinity for cytochrome $c$ in $A. gambiae$ CPR may also possibly relate to a weaker affinity for some cytochrome P450s.

The isolated $A. gambiae$ FAD-binding domain was assessed for its rate of potassium ferricyanide reduction (section 5.2.1.2). Interestingly, the isolated domain was approximately 2-fold more active than the intact enzyme. This suggests some sort of inhibitory effect imparted by the FMN-binding domain with respect to potassium ferricyanide reduction. Potassium ferricyanide receives electrons directly from the FAD cofactor. It is, therefore, possible that there is conformational or spatial crowding in presence of the FMN-binding domain and potassium ferricyanide interaction is optimised in the isolated FAD-binding domain.
When all of the specific activity and kinetic data is considered, it is very difficult to rationalise as the data themselves do not appear to fully explain *A. gambiae* CPR action relative to the human enzyme. The fact that the specific activities of *A. gambiae* and human CPR are practically the same seems contradictory when other data is taken into account. Since *A. gambiae* CPR is deficient in bound flavin cofactors and has an apparent weaker affinity for NADPH it is unclear as to why the activities are so similar. It is eminently possible that because the two CPRs used in this study are from very different sources; insect and mammalian, the CPRs have evolved to act differently due to contrasts in the encountered environments of the two organisms. It is possible that interaction with cytochrome P450 is not the major role of mosquito and instead, interactions with other acceptors such as heme oxygenase (Schacter et al., 1972),

Mutations in human CPR have been shown to have a dramatic effect on the activity of heme oxygenase (Pandey et al., 2010). This observation could also prove to be particularly relevant within blood-sucking insects such as mosquitoes and the relationship between CPR and heme oxygenase in these organisms is of growing importance. The possible shift in focus could have lead to slightly altered structural properties in *A. gambiae* CPR, to optimise interaction with these alternative acceptors, which in turn could explain the flavin
incorporation and NADPH analogue binding data. Whatever the explanation may be, what is abundantly clear is that to fully explain any of the data presented in this thesis, more comprehensive structural determination of *A. gambiae* CPR is required.

To date, the knowledge regarding CPR structure has been determined from X-ray crystallography (Wang *et al*., 1997; Lamb *et al*., 2006) however NMR should not be disregarded as a valuable tool. Although CPR is a very large protein in terms of NMR viability, it has been demonstrated that protein deuteration is effective at improving spectral quality (Figure 3.20). The multi-domain nature of CPR also means NMR has a role to play in biophysical analysis. Taken as separate entities, the isolated FMN and FAD-binding domains are a more manageable size, in particular the FMN-binding domain, and can be structurally assessed to provide valuable information regarding CPR even if the structure of the intact protein is unattainable. In this thesis, the FMN and FAD-binding domains, along with the intact CPR, have been analysed by NMR and significant progress has been made with each. The FMN-binding domain has proved the most successful sample to study, primarily due to its small size and relative stability, with sufficient triple resonance spectra being gathered for backbone assignments. The FAD-binding domain and intact CPR have proved more problematic but both have been deuterated with varying degrees
of success. Although much more work is required, particularly on the higher molecular weight samples, the work to date has been invaluable and provided solid foundations for this more comprehensive study to be carried out.

The structural difference between rat and mosquito CPRs highlighted by the *A. gambiae* CPR model provided a plausible explanation for the low affinity of *A. gambiae* CPR for 2',5'-ADP however the data failed to corroborate the theory. In the absence of a protein structure, mutagenesis is likely to be the most useful tool in the elucidation of further information regarding *A. gambiae* CPR. Despite the T607Q mutant not advancing current knowledge, by mutating further residues known to be involved in either flavin cofactor or NADPH interaction, a vast amount of data could potentially be gathered, even without a fully determined structure.

The work carried out in this thesis has been the first of its kind by focussing on the biochemical and biophysical characterisation of *A. gambiae* CPR. The data gathered has served to highlight differences between the mosquito CPR and the more established human equivalent. The growing number of insecticide resistant strains of mosquitoes has magnified the threat of malarial spread and accelerated the need for the elucidation of novel effective compounds (Ranson *et al.*, 2002). Insecticide detoxification is in part down to the
monooxygenase family of enzymes (Feyereisen, 1999) of which CPR is a prominent member. This work, therefore, has given a valuable insight into the *A. gambiae* CPR enzyme and could serve a crucial role in full structural determination making the production of desired novel compounds imminently possible in years to come.
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Appendix A. MaldiTOF mass spectrometry identification of *A. gambiae* NADPH cytochrome P450 reductase.

Two peptides from a trypsin digested sample of full length *A. gambiae* CPR were identified. Pages 276-277, the fragmentation spectra of the two peptides. Page 278, the BLAST search results for the two peptides and associated hit.
Appendix B. Primer sequences of FMN and FAD-binding domains of A. gambiae CPR. A = FMN-binding domain primers: Red = Forward primer; Brown = Reverse primer #1; Green = Reverse primer #2, Purple = Reverse primer #3. B = FAD-binding domain primers: Red = Reverse primer; Purple = Forward primer #1; Brown = Forward primer #2. Forward primers include an Nco1 restriction site whilst reverse primers include a Kpn1 restriction site for ligation of cloned domain into pETM11 vector.
Appendix C. Intact mass spectrometry of *A. gambiae* FMN-binding and FAD-binding domains. A = FMN-binding domain B = FAD-binding domain. Electrospray mass spectrometry performed at Manchester Interdisciplinary Biocentre.