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NADPH-cytochrome P450 oxidoreductase from the mosquito *Anopheles minimus*: kinetic studies and the influence of Leu86 and Leu219 on cofactor binding and protein stability^s

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Abstract

NADPH-cytochrome *c* oxidoreductase from the mosquito *Anopheles minimus* lacking the first 55 amino acid residues was expressed in *E. coli*. The purified enzyme loses FMN, leading to an unstable protein and subsequent aggregation. To understand the basis for the instability, we constructed single and triple mutants of L86F, L219F, and P456A, with the first two residues in the FMN domain and the third in the FAD domain. The triple mutant was purified in high yield with stoichiometries of 0.97 FMN and 0.55 FAD. Deficiency in FAD content was overcome by addition of exogenous FAD to the enzyme. Both the wild-type and the triple mutant follow a two-site Ping-Pong mechanism with similar kinetic constants arguing against any global structural changes. Analysis of the single mutants indicates that the proline to alanine substitution has no impact, but that both leucine to phenylalanine substitutions are essential for FMN binding and maximum stability of the enzyme.

Cytochromes P450 belong to a superfamily of heme-containing monooxygenases that catalyze the oxidative metabolism of various endogenous and exogenous substrates by inserting one atom of molecular oxygen into substrates. In eukaryotic cells, this reaction occurs in the endoplasmic reticulum and requires electrons from NADPH via its redox partner NADPH-cytochrome P450 oxidoreductase (CYPOR). CYPOR is a membrane-bound protein containing both FAD and FMN cofactors. CYPOR functions as a shuttle transferring 2 electrons, one at a time, from NADPH to different cytochromes P450 [1,2], heme oxygenase [3], cytochrome *b*₅ [4], and fatty acid elongase [5]. CYPOR is also capable of transferring electrons to non-physiological acceptors such as cytochrome *c*, ferricyanide, and menadione *in vitro*.

Malaria is one of the most common vector-borne diseases afflicting millions of people in tropical countries. Reducing the emergence of this disease by use of pyrethroid insecticides is one of the strategies in malaria vector control program. However, pyrethroid resistance mediated by cytochrome P450 enzymes in family 6 (CYP6) has been found in many insect species [6-10]. The mosquito *Anopheles minimus*, one of the primary malaria vectors in Thailand, was previously shown to have increased levels of CYP6AA3 and CYP6P7 transcripts

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during deltamethrin resistance selection [11,12]. Full-length *Anopheles minimus* NADPH-cytochrome P450 oxidoreductase (flAnCYPOR) cDNA has been isolated and expressed in *E. coli* [13]. The purified fl-AnCYPOR enzyme supported metabolism of deltamethrin when reconstituted with membrane fractions of recombinant CYP6AA3. Recently, Lycett et al. [14] have demonstrated that *in vivo* knockdown of CYPOR in *Anopheles gambiae* increases permethrin sensitivity implicating CYPOR in the emergence of drug resistant mosquitoes.

The crystal structure of trypsin-solubilized, *E. coli*-expressed rat CYPOR [15] shows three structural domains: the FMN-binding domain, the connecting domain, and the FAD/NADPH binding domain. In this structure the isoalloxazine ring of FAD is juxtaposed to that of FMN, with the closest distance of ~4Å allowing direct electron transfer between the two flavins (Figure 1). On the basis of steady-state kinetic analysis, various kinetic mechanisms have been proposed for CYPOR including Ping-Pong for pig liver [16], pig kidney [17], house-fly [18], and yeast CYPOR [19]; random sequential for house-fly [20] and *Phanerochaete chrysosporium* CYPOR [21]; and a two-site Ping-Pong mechanism for rat CYPOR [22]. Elucidation of the *Anopheles minimus* CYPOR kinetic mechanism is important for understanding the interaction of this enzyme with its electron transfer partners.

Here we report on the cloning, expression, purification, and characterization of the cytoplasmic portion of *Anopheles minimus* CYPOR ($\Delta 55$ AnCYPOR) protein, which lacks the first 55 residues that comprise the membrane binding region. The isolated protein easily loses its flavin cofactors resulting in an aggregated and inactive protein. In order to understand the basis for this instability and to generate a more stable protein, three residues were chosen for mutagenesis studies: L86, L219, and P456. These residues were selected based on the crystal structure of rat CYPOR (Figure 1) and a sequence alignment of CYPOR proteins from various organisms. Two substitutions, L86F and L219F, were introduced in the FMN binding domain and the third, P456A, was introduced in the FAD binding domain. The purified triple mutant (L86F/L219F/P456F) exhibits increased stability with significant reduction in aggregation compared to the wild-type enzyme. Steady-state kinetic studies of the wild-type and the triple mutant $\Delta 55$ AnCYPOR suggest that both variants have similar kinetic constants and follow the non-classical two-site Ping-Pong mechanism which has been proposed for rat CYPOR enzyme [22]. The higher stability of the triple mutant makes it more suitable for further structural and mechanistic studies. Future structure/function analysis of this mutant and similarly designed full-length AnCYPOR (flAnCYPOR) proteins will help us in our understanding of P450-mediated metabolism of insecticides and will aid in malarial control efforts.

Materials and Methods

Expression & Purification of $\Delta 55$ AnCYPOR

A soluble form of *Anopheles minimus* CYPOR ($\Delta 55$ AnCYPOR) was constructed by deleting the first 55 amino acids by PCR using fl-AnCYPOR cDNA [13] as a DNA template. The upstream primer (5'-AGTCTAGCTCATATGTCGATCCAGCCGACCACGGTAAAC-3') contained an *NdeI* restriction site. A T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') which contained a *HindIII* site was used as the downstream primer. After amplification and digestion, $\Delta 55$ AnCYPOR cDNA was ligated into the unique *NdeI/HindIII* sites of the expression plasmid pET-28a (Novagen, Madison, WI), which contains an N-terminal 6×his-tag and a thrombin cleavage site. The DNA sequence of the construct was verified, and the plasmid was transformed into BL21(DE3) cells for expression.

A culture was grown in LB media containing 50 mM potassium phosphate, pH 7.0, 4% glycerol, 100 μ M riboflavin, and 15 μ M/ml kanamycin at 37°C to an OD₆₀₀ of 0.1-0.2 when protein expression was induced by addition of 0.1 mM IPTG. The cells were grown overnight at 16°C after induction and harvested by centrifugation at 5000×g for 10 min. After resuspension in

binding buffer (50 mM Tris pH 7.7, 0.1 M NaCl, 10% glycerol, and 10 mM imidazole), the cells were lysed by sonication. The lysate was centrifuged at 100,000×g for 60 min and the supernatant was applied to a Ni²⁺-NTA affinity column (Qiagen, Valencia, CA) previously equilibrated with the binding buffer. The column was extensively washed with binding buffer followed by the same buffer containing 20 mM imidazole. The protein was eluted by increasing the imidazole concentration from 50 mM to 200 mM. Purification of the soluble Δ55AnCYPOR protein was monitored by analysis of fractions by SDS-PAGE analysis. Pure fractions were pooled, concentrated and further purified by HPLC (Shimadzu Scientific Instruments) using a Superdex 200 column (Amersham Bioscience). The column was run in a buffer solution containing 20 mM Tris-HCl, pH 8.0 and 20 mM NaCl, at flow rate of 0.2 ml/min. The purity of protein was assessed by SDS-PAGE. Protein concentration was determined by the Bio-rad protein assay (Bio-rad, Hercules, CA) using soluble rat CYPOR protein as a standard. Thrombin cleavage of purified Δ55AnCYPOR was performed in order to study if the N-terminal his-tag affected the properties of this enzyme. After digestion of the enzyme by thrombin at 4°C, the reaction mixture was passed through Ni²⁺-NTA affinity column. The flow through containing the untagged protein was collected, concentrated and stored at -80°C until use.

Site-Directed Mutagenesis of Δ55AnCYPOR Construct, Expression, and Purification

Mutagenesis of the wild-type Δ55AnCYPOR construct was performed to produce 3 separate mutations at amino acid positions 86, 219, and 456 using the Quickchange PCR mutagenesis kit (Stratagene, LaJolla, CA). The primer pair for each mutant is listed below; L86F (5'-CCGTTTGGTGGTGGTCTACGGTCCC-3' and 5'-GGGAACCGTAGAACACCACCAACGG-3'); L219F (5'-GAACATTGAGGATTACTTCATCACGTGGAAGG-3' and 5'-CCTTCCACGTGATGAAGTAATCCTCAATGTTC-3'); P456A (5'-GTTGCCCCGCCTGCAGGCCGCTACTACTC-3' and 5'-GAGTAGTAGCGGCCTGCAGGCGGGGCAAC-3'). For creating the triple mutant, plasmids from L86F, L219F and P456A were digested with *NdeI-SacI*, *SacI-PstI*, and *PstI-HindIII* respectively. The DNA fragments containing individual mutations were purified and ligated into *NdeI-HindIII* sites of pET-28a vector. The constructs were verified by DNA sequencing and transformed into BL21(DE3) cells. The three single mutants were expressed under the same conditions as the wild-type enzyme. The triple mutant (L86F/L219F/ P456A) was grown in modified TB (10 g tryptone and 20 g yeast extract in 1 litre of 50 mM potassium phosphate, pH 7.0, 4% glycerol) media containing 100 μM riboflavin and 15 μg/ml kanamycin. The culture was grown at 37°C until its OD₆₀₀ was 0.8-1.0 when 0.4 mM IPTG was added followed by 24 hrs of incubation at 25°C. All of the mutant proteins were purified using the same protocol developed for the wild-type enzyme (see above).

Measurement of Flavin Contents

FAD and FMN contents of each sample were measured by using a fluorometric method as described previously [23]. Commercial FMN and FAD were further purified by the HPLC method [23] and used for the standard curve and for cofactor supplementation experiments. The Bio-rad protein assay (Bio-rad) was utilized to determine the concentration of protein using purified rat CYPOR protein as standard. The concentrations of standard FAD and FMN solutions were determined spectrophotometrically at 450 nm using extinction coefficients of 11.3 and 12.2 mM⁻¹cm⁻¹, respectively [23].

Spectrophotometric Methods and Activity Assays

All spectrophotometric measurements were performed using a Shimadzu UV-2501PC spectrophotometer. The FAD-reconstituted enzyme was diluted and concentrated using

Centriprep (Millipore) to remove unbound FAD before taking a spectrum. The oxidized enzyme was then diluted in 300 mM potassium phosphate buffer, pH 7.7 to a final concentration of 10-15 μM and the spectrum was recorded. Another spectrum was obtained after addition of NADPH (300 μM final concentration) under aerobic conditions.

The CYPOR-mediated cytochrome *c* reduction was carried out in 300 mM potassium phosphate buffer, pH 7.7 as described by Shen *et al*, 1989 [24] with minor modifications. After 1 min pre-incubation of enzyme in buffer with 50 μM cytochrome *c* at 25°C, the reaction was initiated by addition of 50 μM NADPH. NADPH-dependent cytochrome *c* reduction was followed by a change in the absorbance at 550 nm. The ferricyanide reduction was performed in 50 mM potassium phosphate buffer, pH 7.7 containing 1 mM ferricyanide and 50 μM NADPH at 25 °C [25]. The reduction of ferricyanide was monitored by a change in absorbance at 420 nm with an extinction coefficient of $1.02 \text{ mM}^{-1}\text{cm}^{-1}$ [25]. Velocities are expressed as $\mu\text{mol/minute/mg}$ protein.

Steady-state kinetic studies of cytochrome *c* reduction were performed in 300 mM potassium phosphate buffer pH 7.7 at 25 °C in a final volume of 0.5 ml. Substrate saturation experiments were performed by varying cytochrome *c* concentration at four different fixed NADPH concentrations (5, 10, 25, and 50 μM). The reaction was started by addition of the enzyme (4-10 nM) supplemented with 10 fold excess FAD. The initial velocity data were analyzed by non-linear regression and fit to an equation for Ping-Pong mechanism by using the Grafit 6.0 software package..

For inhibition studies with NADP^+ and 2'-AMP, cytochrome *c* reduction assays were performed as described above. The reaction mixtures contained different concentrations of NADPH and a constant concentration of cytochrome *c* (35 μM) in the absence and presence of NADP^+ (0, 50, 100, and 200 μM) or 2'-AMP (0, 50, 100, and 400 μM). Inhibition constants were derived from Dixon-plot using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego CA).

Results and Discussion

Expression and Purification of $\Delta 55\text{AnCYPOR}$

Detailed biochemical and structural studies of full length AnCYPOR (flAnCYPOR) have been hampered by poor yields of the active protein, due to the protein expression as inclusion bodies in *E. coli*. Therefore, a soluble form of AnCYPOR ($\Delta 55\text{AnCYPOR}$) was constructed by deletion of the first 55 amino acid residues, which comprise the membrane binding region based on the amino acid sequence alignment and the crystal structure of rat CYPOR [15], and expressed in *E. coli*. Although a large portion of expressed $\Delta 55\text{AnCYPOR}$ formed inclusion bodies, the amount of soluble AnCYPOR isolated from this construct was significantly higher than that from flAnCYPOR. An extensive screening of different expression conditions [26-28] was conducted to maximize production of this enzyme in a more soluble form. Protein expression at an OD_{600} of 0.1-0.2 with 0.1 mM IPTG at 16° C in modified LB media (containing 50 mM phosphate buffer, pH 7.0 and 4% glycerol) resulted in a sufficient amount of the soluble protein for protein isolation and subsequent characterization.

Recombinant $\Delta 55\text{AnCYPOR}$ was purified by Ni-agarose affinity chromatography followed by gel filtration using superdex-200 column. The purified enzyme eluted from Ni-column at 50 mM imidazole. This purification step separated the yellow (flavinated) $\Delta 55\text{AnCYPOR}$ from the colorless (flavin-free) $\Delta 55\text{AnCYPOR}$ protein, as the latter binds more tightly to the Ni-resin compared to the former. Supplementing growth media with riboflavin did not improve production of flavin-bound enzyme. Yellow colored fractions with high purity as determined by SDS-PAGE were pooled, concentrated and applied to the superdex-200 column which

helped in removing the aggregated form of the enzyme. Purification by gel filtration resulted in a homogeneous preparation of 73 kDa enzyme as anticipated (data not shown). Our results indicate a recovery 2 mg of purified enzyme per liter of culture (data not shown).

Flavin Contents and Protein Stability of $\Delta 55\text{AnCYPOR}$

Purified $\Delta 55\text{AnCYPOR}$ enzyme contained 0.51 ± 0.07 mol of FMN and 0.63 ± 0.02 mol of FAD per mol of protein (Table 1). The stability of the isolated enzyme was tested by performing limited proteolysis with trypsin. Treatment of purified $\Delta 56$ rat CYPOR (Figure 2A) and wild-type $\Delta 55\text{AnCYPOR}$ (Figure 2B) with trypsin showed a difference in time dependent proteolysis of each enzyme as indicated by SDS-PAGE. $\Delta 55\text{AnCYPOR}$ showed significant degradation after 5 minutes of digestion, as compared to $\Delta 56$ rat CYPOR, which showed almost no degradation by the end of 2 hours, indicating that $\Delta 55\text{AnCYPOR}$ is significantly less stable than $\Delta 56$ rat CYPOR. It is interesting to note that at the end of 5 min of digestion of $\Delta 55\text{AnCYPOR}$, there is an accumulation of a 50 kDa band corresponding to FAD domain but no accumulation of the 25 kDa FMN domain (Figure 2B). This suggests that the FMN domain is relatively more unstable in this enzyme. Supplementing the enzyme with FMN did not stabilize the protein, suggesting that the FMN-deficient protein may have a sufficiently different fold from the intact enzyme such that FMN cannot be reincorporated into its binding site. This behavior is in contrast to the ratCYPOR in which exogenous FMN can be easily incorporated into its FMN binding site [32].

Activities and Spectral Features of $\Delta 55\text{AnCYPOR}$

The specific activity of the purified $\Delta 55\text{AnCYPOR}$ is $8.7 \mu\text{mol/min/mg}$ (Table 2). The addition of 10-fold excess FAD increased the cytochrome *c* reduction activity by 1.6 fold to $14.4 \mu\text{mol/min/mg}$ (Table 2). An increase in the activity of $\Delta 55\text{AnCYPOR}$ with the addition of exogenous FAD was not anticipated as FMN occupancy was 0.51 ± 0.07 and FAD occupancy was 0.63 ± 0.02 mol per mol of protein (Table 1). It is possible that there is a small population of enzyme in which FMN occupies the FAD binding site, as FMN is smaller than FAD and has some of the same binding determinants as FAD. Another, a more likely possibility is that there are enzyme molecules that have either only FAD or only FMN, and the species that lacks FAD (i.e., containing only FMN) accepts exogenous FAD and becomes functional. The specific activity of $\Delta 55\text{AnCYPOR}$ ($14.4 \mu\text{mol/min/mg}$) is comparable to values for the mammalian enzymes (ranging from $27\text{--}52 \mu\text{mol/min/mg}$) but almost 18 fold higher than that for fl-AnCYPOR ($0.77 \mu\text{mol/min/mg}$) [13]. The reported values for fl-AnCYPOR may be lower partly due to the fact that 100 mM Tris-HCl was used in their assays, whereas 300 mM phosphate was employed in our $\Delta 55\text{AnCYPOR}$ and mammalian CYPOR assays. A number of reports have demonstrated the effect of ionic strength on activity of CYPOR and interactions of both substrates with CYPOR [20,31,33,34]. According to these reports, enzyme activity increases by 2-3 fold with increase in ionic strength, because dissociation of NADP^+ becomes rate limiting at low ionic strength. At higher ionic strength a conformational change in the enzyme has been proposed to be the rate limiting step [33,34]. However, the difference in ionic strength accounts for only 3-4 fold difference between the activities of full-length and $\Delta 55\text{AnCYPOR}$. Another 4-5 fold difference may be due to the absence of membrane binding region and/or a higher purity of $\Delta 55\text{AnCYPOR}$ enzyme preparation. Ferricyanide reduction activities were measured to assess electron transfer from NADPH to FAD without involvement of FMN. The specific activity of this enzyme in the presence of 10X FAD for ferricyanide reduction was $28.7 \mu\text{mol/min/mg}$ (Table 2) which is 48 % and 28 % of the corresponding values of housefly fl-CYPOR ($59.2 \mu\text{mol/min/mg}$) [18] and rat CYPOR ($102 \mu\text{mol/min/mg}$) [24], respectively. The spectrum of the purified $\Delta 55\text{AnCYPOR}$ is typical for oxidized flavoproteins (Figure 3A, spectrum 1). Addition of NADPH under aerobic conditions reduced the enzyme and produced the air-stable semiquinone with a characteristic broad peak around 500-650 nm (Figure 3A, spectrum 2).

The N-terminal his-tag of $\Delta 55$ AnCYPOR was removed by thrombin treatment and the enzyme without the tag was isolated. Characterization of the untagged enzyme showed that its specific activity and flavin contents were indistinguishable from his-tagged $\Delta 55$ AnCYPOR (data not shown). Therefore his-tagged protein was used throughout this study.

Expression and Purification the Triple Mutant, L86F/L219F/P456A

A loss of both flavin cofactors and the instability of $\Delta 55$ AnCYPOR protein prompted us to search for differences in key amino acid residues between $\Delta 55$ AnCYPOR and CYPOR proteins from other organisms. Amino acid sequence alignment (Figure 4) indicates that, in the FMN domain of $\Delta 55$ AnCYPOR, amino acids at positions 86 and 219 are leucines, while there are highly conserved phenylalanines at both positions in other CYPOR sequences. Although these two phenylalanines are not in direct contact with FMN as indicated in the rat CYPOR structure [15], they are deeply buried in two hydrophobic cores (one formed by F69, F83, F135, and F152; the other by F94, F171, F216 and W219) of the FMN domain (Figure 1), suggesting that these two amino acids are likely to be involved in protein folding and stabilization of the FMN-binding domain. The amino acid at position 456 is proline in AnCYPOR, whereas the corresponding amino acid in house fly and mammalian CYPORs is an alanine. Proline 456 is located in the FAD domain and could change the FAD-binding properties because of the restricted conformational flexibility. Thus, these substitutions, L86F, L219F, and P456A, were made with the goal of making a more stable $\Delta 55$ AnCYPOR.

The triple mutant did not require growth at low temperature. The mutant could be expressed at high levels under a variety of conditions without significant inclusion body formation. Purification of the mutant by the nickel column showed that less than 2% of total eluted protein was flavin-free as compared to 20% in the case of the wild-type enzyme (data not shown). In addition, no high-order oligomers of the triple mutant were detected by gel filtration chromatography (data not shown). The triple mutant was purified in high yield (10 mg/liter), an improvement of 5 fold over wild type $\Delta 55$ AnCYPOR.

Flavin Content and Stability of L86F/L219F/P456A

The flavin content analysis shows that the triple mutant contains 0.97 ± 0.02 mole of FMN per mol of protein as compared to 0.51 ± 0.07 for $\Delta 55$ AnCYPOR but FAD content remains similar (Table 1). This indicates that, unlike the wild-type enzyme the triple mutant does not lose FMN during purification. Limited tryptic proteolysis of the triple mutant (Figure 2C) showed that the ~73 kDa band (the intact protein) persisted after 2 hrs whereas the same band representing the intact enzyme disappeared in less than one hour in the wild-type enzyme (Figure 2B), indicating a greater stability of the triple mutant. There is an accumulation of a band at ~50kDa corresponding to the FAD domain in both enzymes, while the FMN domain (~25 kDa) of wild-type $\Delta 55$ AnCYPOR, is quickly digested into smaller fragments. In contrast, the triple mutant shows an accumulation of the 25kDa band, suggesting that the FMN domain in the triple mutant is more stable than that of the wild-type enzyme. Furthermore, the FAD-supplemented triple mutant stays intact after 2 hrs of trypsin digestion under the same conditions (data not shown), almost as stable as the rat enzyme (Figure 2A).

Activities and Spectral Features of L86F/L219F/P456A

The triple mutant shows a modest increase in specific activity as compared to the wild-type enzyme toward both cytochrome *c* and ferricyanide (Table 2). However, addition of exogenous FAD increased specific activities by 2-3 fold to 31.3 $\mu\text{mol/min/mg}$ when cytochrome *c* was used as the electron acceptor and 52.6 $\mu\text{mol/min/mg}$ when ferricyanide was used. Both activities are comparable to those of house-fly CYPOR (cytochrome *c*, 32.5 $\mu\text{mol/min/mg}$; ferricyanide, 59.2 $\mu\text{mol/min/mg}$) [20,18] and rat (cytochrome *c*, 51.5 $\mu\text{mol/min/mg}$; ferricyanide, ~102 $\mu\text{mol/min/mg}$) [24]. A 2-fold increase in activity is consistent with an almost

stoichiometric occupancy of FMN site and exogenous FAD occupying vacant FAD site in the triple mutant. As seen with the wild type enzyme, it is conceivable that there is a small population of the mutant enzyme containing FMN in the FAD binding site that accounts for additional stimulation when displaced by exogenous FAD. The absorbance spectrum of the triple mutant of $\Delta 55\text{AnCYPOR}$ was typical for oxidized CYPOR enzymes (Figure 3B, spectrum 1). As with the wild type enzyme, upon addition of NADPH under aerobic conditions, the protein was reduced and showed the formation of an air-stable semiquinone species with a characteristic broad peak around 500-650 nm (Figure 3B, spectrum 2). The triple mutant shows formation of more semiquinone than the wild-type enzyme which is consistent with a higher stoichiometry of FMN in the triple mutant (0.97 ± 0.02), compared to the wild-type enzyme (0.51 ± 0.07).

Kinetic Characterization of Wild Type and the Triple Mutant

A detailed kinetic characterization was performed to verify that the mutations did not result in any drastic changes in the triple mutant. Both wild-type and triple mutant of $\Delta 55\text{AnCYPOR}$ catalyzed NADPH-dependent cytochrome *c* reduction following Michaelis-Menten kinetics with respect to both substrates (cytochrome *c* and NADPH). Preliminary kinetic analysis of wild-type $\Delta 55\text{AnCYPOR}$ indicated a ~1.5 fold increase in the specific activity of the enzyme in the presence of exogenous FAD. However, there were no significant differences in the apparent K_m values for both substrates (data not shown), suggesting that an increase in activity is due to insertion of FAD into the FAD binding site and not due to changes in the binding mode or binding sites of the substrates. Thus additional steady state kinetics was performed using an enzyme to which 10 fold excess FAD had been added. Detailed kinetic analysis was performed by measuring initial velocities of cytochrome *c* reduction activity as a function of cytochrome *c* or NADPH concentrations at a fixed concentration of the second substrate. The initial velocity data fit best to the equation for a classical one-site Ping-Pong mechanism [29], previously proposed for pig liver [16], pig kidney [17], and house fly CYPOR [18]. The kinetic constants obtained by non linear regression analysis of data are listed in Table 3. K_m values for both cytochrome *c* (37.3 μM) and NADPH (19.2 μM) are approximately an order of magnitude higher than the corresponding K_m values for fl-AnCYPOR (cytochrome *c*, 1.24 μM ; NADPH, 2.58 μM) [13] and fl-housefly CYPOR (cytochrome *c*, 4.6 μM ; NADPH, 0.96 μM) [18]. Again, this is consistent with published reports [20,31,33,34] demonstrating that higher ionic strength is correlated with weaker binding of both substrates. Phosphate may also have additional inhibitory effects on NADPH binding, as Muratiliiev *et al.* [20] documented that inorganic phosphate acts as an inhibitor ($K_i = 33.3 \text{ mM}$) of housefly enzyme competitive with respect to NADPH. K_m of $\Delta 55\text{AnCYPOR}$ is much closer to K_m for human (NADPH, 15.6 μM) [30] and rat CYPOR (cytochrome *c* 21.2 μM ; NADPH, 6.4 μM) [24], where activities were measured in the presence of 300 mM phosphate buffer. These observations underscore the importance of ionic interactions between both substrates and CYPOR. The three-dimensional structure of rat CYPOR indicates the presence of a negatively charged surface of the FMN domain which has been proposed to be the binding site for the positively charged surface of cytochrome *c* [15], in addition to the presence of positively charged residues in the NADPH binding site of CYPOR. Site-directed mutagenesis results confirm the role of the negatively charged surface residues of rat CYPOR in interaction with cytochrome *c* [35]; and the positively charged residues in interaction with NADPH binding [36].

Detailed kinetic analysis of the triple mutant was also performed to test if the three engineered substitutions resulted in any changes in the reaction mechanism. Similar analysis by fitting the data to an equation for Ping-Pong mechanism indicates that the triple mutant follows the same kinetic mechanism as the wild-type $\Delta 55\text{AnCYPOR}$. The cytochrome *c* K_m for the triple mutant is 92.4 μM and the NADPH K_m is 42.5 μM compared to 37.2 μM and 19.2 μM of the corresponding values, respectively, for the wild-type enzyme indicating that the mutations did

not significantly alter the structure or substrate binding mode of the mutant. It is interesting to note that the turnover number for the triple mutant is 69.7 s^{-1} , which is almost 3-fold higher than that for wild-type $\Delta 55\text{AnCYPOR}$ (Table 3) and is comparable to those of the human and rat enzymes. The difference in activities between the wild type and the mutant can be ascribed to a 1.9 fold higher stoichiometry of FMN in the triple mutant (Table 1).

The apparent K_m for NADH is 8.4 mM for the wild-type enzyme and 10.8 mM for triple mutant enzyme, about 500 fold higher than the K_m for NADPH (Table 3), thus demonstrating the importance of 2'-phosphate of NADPH in interaction with AnCYPOR as observed with CYPORs from other sources. This again indicates that the binding mode for NADPH in $\Delta 55\text{AnCYPOR}$ and the triple mutant is similar to that in other CYPORs and that the mutations did not cause any changes in the NADPH binding mode in the triple mutant of $\Delta 55\text{AnCYPOR}$.

Inhibitions by NADP^+ (product) and 2'-AMP (dead-end inhibitor) were studied. Initial velocities were measured as a function of NADP^+ or 2'-AMP concentration at different NADPH concentrations. The data were best fit to an equation for competitive inhibition indicating that both NADP^+ and 2'-AMP act as competitive inhibitors with respect to NADPH. The K_i value for NADP^+ for the wild-type is 32.2 μM (Table 3) compared to 18.8 μM for rat fl-CYPOR under similar assay conditions [31]. A K_i of 71.1 μM for 2'-AMP for wild-type $\Delta 55\text{AnCYPOR}$ is comparable to 187 μM for house fly fl-CYPOR [18]. The triple mutant of $\Delta 55\text{AnCYPOR}$ shows comparable K_i values of 45.9 μM for NADP^+ and 131.9 μM for 2'-AMP indicating the structural integrity of the mutant.

The classical one-site Ping-Pong mechanism [37] predicts inhibition by NADP^+ that is competitive with cytochrome c^{3+} and noncompetitive with NADPH; and inhibition by cytochrome c^{2+} that is competitive with NADPH and noncompetitive with cytochrome c^{3+} . However our results indicate that NADP^+ and 2'-AMP act as competitive inhibitor with respect to NADPH in both the wild-type and triple mutant, in agreement with reports on other CYPOR enzymes. Observed initial velocity pattern along with the product inhibition by NADP^+ and dead-end inhibition by 2'-AMP in wild-type and the triple mutant suggests that, instead of classical one-site Ping-Pong mechanism, AnCYPOR follows a two-site Ping-Pong mechanism [37] as reported for rat CYPOR [22].

Single Mutants

In order to identify the roles of the three mutated amino-acids in stabilization of the triple mutant, single mutants were generated and purified. L86F and L219F proteins are indistinguishable in their flavin contents (Table 1) and specific activities (Table 2). Our results indicate that the FMN contents of purified L86F and L219F are both 0.83 mol of FMN/mol protein which is somewhat less than 0.97 mol of FMN/mol protein for the triple mutant but significantly more than 0.51 mol of FMN/mol protein for wild type. The addition of FAD increased the specific activity of L86F and L219F from $\sim 8.1 \mu\text{mol/min/mg}$ to $\sim 19.6 \mu\text{mol/min/mg}$, whereas the specific activity of the triple mutant protein increased from $\sim 10.2 \mu\text{mol/min/mg}$ to $\sim 30.5 \mu\text{mol/min/mg}$. Similar increases were observed when ferricyanide was used as the electron acceptor. These observations suggest that each phenylalanine contributes significantly toward FMN retention, but substitution of both leucine residues to phenylalanine is essential for maximum flavin content and activity. Although P456 is located in the FAD binding region, substitution of this residue did not improve FAD stoichiometry (Table 1). Instead P456A showed a modestly lower FMN stoichiometry (0.3) than that of the wild-type enzyme (0.5). Both the wild-type enzyme and P456A exhibited further loss of FMN upon storage, indicating that their stabilities could be ranked as equivalent. Therefore, it is reasonable to conclude that the proline replacement does not have a significant effect on the triple mutant (L86F/L219F/P456A).

The three-dimensional structure of rat CYPOR indicates that the phenylalanines corresponding to leucines at positions 86 and 219 of AnCYPOR are stacked against several aromatic residues buried in two extremely hydrophobic cores. The beneficial effects of leucine replacements by phenylalanine on the stability of the triple mutant suggest that the two phenylalanines may assist in folding and stabilization of the FMN domain. It is not clear why leucines are the amino acid of choice by nature at positions 86 and 219 in wild-type AnCYPOR. Despite the near stoichiometric amount of FMN in the triple mutant, the FAD content remains at 63%. It is apparent that a low stoichiometry of FAD may not matter much in this enzyme, at least *in vitro*, as FAD can be easily reconstituted into the FAD binding site of AnCYPOR, unlike CYPORs from other organisms. The structural basis for the loose binding of FAD to the mosquito CYPOR must await its three-dimensional structure analysis.

In summary, we have generated a more soluble and stable form of AnCYPOR which is similar in its spectral and kinetic properties to the wild-type enzyme. We conclude that the triple mutant (L86F/L219F/P456A) or a double mutant (L86F/L219F) will serve as a good model of mosquito CYPOR in future structural studies and in the development of new antimalarial agents.

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Figure 1. The crystal structure of rat CYPOR showing the locations of residues corresponding to the mutation sites in the AnCYPOR triple mutant (L86F/L219F/P456A)

The three substitution sites are marked with black balls and the vicinities of the two phenylalanine substitutions (circled) are enlarged to illustrate the stacking of the hydrophobic residues. Residue F83 (L86 of AnCYPOR) is packed against the aromatic rings of F69, F135, and F 152; and F216 (L219 of AnCYPOR) is stacked against F94, F171 and W 219. A453 corresponding to P456 of AnCYPOR is located in the FAD domain. The three structural domains of CYPOR are shown: the FMN domain (pink), connecting domain (blue) and the FAD domain (green). The cofactors are represented as stick models: FMN (green), FAD (yellow) and NADP⁺ (black).

Figure 2. SDS-PAGE showing limited tryptic proteolysis of purified rat CYPOR (A), wild-type $\Delta 55\text{AnCYPOR}$ (B), and the triple mutant (C)
The reactions were performed at room temperature and stopped at indicated time points with soybean trypsin inhibitor. Lane M is for the molecular weight marker. The triple mutant is considerably more stable than the wild type mosquito enzyme. However, both mosquito enzyme variants are still less stable than the rat enzyme.

Figure 3. Absorbance spectra of wild-type $\Delta 55$ AnCYPOR (A) and the triple mutant (B)
 Spectrum 1 in each panel was obtained with 10-14 μ M oxidized $\Delta 55$ AnCYPOR enzyme in 0.3 M potassium phosphate buffer, pH 7.7. In each case, the enzyme was reduced by addition of NADPH (300 μ M) under aerobic conditions to form air-stable semiquinone shown in spectrum 2.

Figure 4. Sequence alignment of segments of NADPH-cytochrome P-450 oxidoreductases from various organisms

The arrows indicate the positions of amino acids in the *An. minimus* enzyme and the residue numbers of the other sequences are shown at the right side of each sequence. The amino acids different from the *An. minimus* sequence are in bold letters. The three mutated residues are highlighted.

Table 1Flavin Content^a of Wild Type and Mutant $\Delta 55\text{AnCYPOR}$

Enzyme	FMN	FAD
<i>Rat CYPOR</i>	0.92 ± 0.01	1.05 ± 0.05
<i>$\Delta 55\text{AnCYPOR}$</i>	0.51 ± 0.07	0.63 ± 0.02
<i>Triple Mutant</i>	0.97 ± 0.02	0.55 ± 0.04
<i>L86F</i>	0.83 ± 0.03	0.50 ± 0.04
<i>L219F</i>	0.83 ± 0.03	0.53 ± 0.01
<i>P456A</i>	0.28 ± 0.02	0.50 ± 0.01

^aThe flavin content is expressed as mol of flavin per mol of protein.

Table 2Specific Activity of Wild-type and Mutant $\Delta 55\text{AnCYPOR}$ with Cytochrome *c* and Ferricyanide as Electron Acceptor

Enzyme	Cytochrome <i>c</i> reduction ^a		Ferricyanide reduction ^a	
	– FAD	+ FAD	– FAD	+ FAD
$\Delta 55\text{AnCYPOR}$	8.7 ± 0.3	14.4 ± 0.1	19.7 ± 0.2	28.7 ± 0.46
<i>Triple Mutant</i>	10.4 ± 0.3	31.3 ± 0.20	25.5 ± 0.4	52.6 ± 1.65
<i>L86F</i>	8.1 ± 0.2	19.8 ± 0.5	18.7 ± 0.2	38.9 ± 0.30
<i>L219F</i>	8.0 ± 0.2	19.3 ± 0.1	19.4 ± 1.4	37.2 ± 0.9

^aThe specific activity is expressed as μmol of substrate reduced/min/mg of protein. The data shown are the average of triplicate measurements.

Table 3Kinetic Constants for Cytochrome *c* Reduction by Wild Type and the Triple Mutant of $\Delta 55\text{AnCYPOR}^a$.

Kinetic constant	wt $\Delta 55\text{AnCYPOR}$	L86F/L219F/P456F
$V_{\max}$, s^{-1}	23.8 ± 1.3	69.7 ± 7.5
K_m for NADPH, μM	19.2 ± 1.7	42.5 ± 6.1
K_m for cytochrome <i>c</i> , μM	37.3 ± 0.2	92.4 ± 13.8
K_i for NADP ⁺ , μM	32.2 ± 3.1	45.9 ± 3.2
K_i for 2'AMP, μM	71.1 ± 8.0	131.9 ± 13.8
K_m for NADH, mM	8.4 ± 0.1	10.8 ± 0.3
$V_{\max}$ (NADH), s^{-1}	8.9 ± 0.1	5.9 ± 0.1

^a The values are obtained from steady-state kinetic studies as described in Materials and Methods.