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Abbreviations: BMR: Reductase domain of cytochrome P450 BM3; CPR: NADPH-cytochrome P450 reductase; FAD: Flavin adenine dinucleotide; FMN: Flavin mononucleotide; NADPH: β-Nicotinamide adenine dinucleotide phosphate.

Abstract Understanding the uncoupling at the heme active site and/or at the level of multi-domain electron transfer is an important element in cytochrome P450 chemistry. Here a chimeric model system consisting of human cytochrome P450 3A4 and the soluble reductase domain of CYP102A1 from *Bacillus megaterium* (P450 3A4-BMR) is used to study the relationship between electron transfer and the coupling efficiency in substrate monoxygenation. Several regulatory features were considered. FAD and FMN added to apo-enzyme in over-saturating concentrations neither influence formaldehyde production nor coupling efficiency. Optimal conditions of coupling efficiency depended only on the NADPH concentration. The pH (pH 8.0) and ionic strength (50 mM potassium phosphate) were found to modulate the level of coupling indicating an influence over the formation of a productive interaction(s) between the BMR and the heme domains. Overall uncoupling is found to be an intrinsic property of the heme-domain, and the covalent linkage of the reductase in a single polypeptide chain has little influence over the activity coupled to product formation.

Keywords: Uncoupling · P450 fusion protein · Molecular Lego approach · cytochrome P450 BM-3 · erythromycin · electron transfer · demethylation

Introduction

Cytochromes P450 are heme-thiolate monooxygenases found in most organisms and they catalysed the NADPH-dependent monooxygenation of different xenobiotic and endobiotic lipophilic substrates [1]. Their function relies on a redox chain that involves flavo- and/or iron-cluster proteins that mediate the transfer of electrons from NADPH or NADH to the heme. One important factor for their enzymatic function is uncoupling, a process that causes a deviation of electrons provided by NADPH from the monoxygenation of the substrate to the formation of superoxide anion radical, hydrogen peroxide and water [2-4]. The uncoupling in human P450 3A4 can be as high as 85-95% [5] and it can occur in the heme reaction cycle at three branch points. The first is the formation of the anion superoxide due to the decay of the superoxo-ferrous ($[FeO_2]^{2+}$-RH). The second is the release of hydrogen peroxide that involves the protonation of the intermediate to peroxy-ferric ($[FeO_2H]^{2+}$-RH). The third branch involves the release of an oxygen atom in the form of a water molecule and it requires the addition of two more electrons following the decay of the oxo-Fe(IV)-porphyrin-p-cation [6].

A key issue in P450 function is to determine whether the uncoupling occurs during the electron flow through the multiple redox centres embedded in the protein matrix, or at the heme-iron active site. In this paper, we address this issue by studying an engineered model system consisting of a chimera between human cytochrome P450 3A4 and the reductase of cytochrome P450 BM3 from *Bacillus megaterium* (BMR), previously developed in our laboratory [7, 8]. Our investigation starts by studying the electron flow through the multiple redox centres and goes to explore possible parameters (flavins, substrate and NADPH concentration, pH and ionic strength) influencing its functionality.

Cytochrome P450 3A4 (CYP3A4) is the most abundant P450 in human liver. It is responsible for phase I metabolism of more than 50% of the currently marketed pharmaceuticals [9, 10]. Its redox

partner, the NADPH-dependent reductase (CPR), is a 76.5 kDa single polypeptide flavoprotein that binds one molecule each of the flavin cofactors FMN and FAD [11, 12] and shares a 38% sequence identity with BMR. Kinetic, spectroscopic and potentiometric studies using a reconstituted liver microsomal monooxygenase system indicate that the hydride ion is transferred from NADPH to the lower redox potential FAD that then transfers single electrons to FMN, which in turn reduces the catalytic heme centre of cytochrome P450 [13]. Fine molecular mechanisms, such as substrate binding [14] and interaction with the redox partner [15-20] have been shown to be important for productive turnover of the enzyme. As a matter of fact, in the bacterial cytochrome P450 BM3 from *Bacillus megaterium*, where a NADPH-dependent reductase domain (BMR) is naturally fused to the heme-containing catalytic domain, displays the highest catalytic efficiency and a nearly complete coupling of the reducing equivalents for substrate monoxygenation [21-23]. This suggests that the interaction between P450s and their redox partner is essential to control catalysis and uncoupling.

The question we address here is whether and, if so, to which extent the uncoupling is due to the interaction between redox partners or intrinsic to the heme. In other words, is it possible to control the level of uncoupling and substrate monoxygenation by optimizing the electron flow from NADPH to heme?

Materials and Methods

Chemicals

FAD (Riboflavin 5-adenosine diphosphate disodium salt), FMN (Riboflavin 5-phosphate sodium salt), erythromycin, horseradish peroxidase type X, superoxide dismutase from bovine erythrocytes, N,N-dimethylaniline and 4-amino-2,3dimethyl-1-phenyl-3-pyrazolin-5-one were purchased from Sigma. NADPH (β-nicotinamide-adenine-dinucleotide phosphate, reduced, tetra sodium salt) was acquired from Calbiochem. All other chemicals were purchased from Sigma-Aldrich at the highest commercially available grade.

Preparation of P450 3A4-apoBMR

The recombinant P450 3A4-BMR was heterologously expressed in *E. coli* strain DH5α from the plasmid pCW-CYP3A4-BMR. Expression, purification and enzymatic activity of the holo-enzyme (120.5 kDa) were performed according to the methods previously described [7].

P450 3A4-BMR in the form of apo-flavin enzyme (P450 3A4-apoBMR) was obtained by extensively washing the fusion protein eluted and collected from hydroxyapatite column (in the last step of purification) with deflavination-buffer containing 200mM KCl, 20% glycerol in 500 mM potassium phosphate buffer at pH 8.0, using a centrifugal concentrating device with 50 kDa cut-off membrane (Vivaspin).

This high salt concentration resulted in the total lost of both FAD and FMN. At the end of this procedure the concentration of P450 3A4-apoBMR was estimated spectrophotometrically by the method of Omura and Sato [24] based on reduced CO difference spectra using an extinction coefficient of ε_{450} of 91 $mM^{-1}cm^{-1}$.

The purity of P450 3A4-apoBMR estimated by SDS-polyacrylamide gel electrophoresis showed a molecular weight of about 119 kDa, in keeping with the fact that our fused protein was depleted in flavins. The residual content of flavins was determined using fluorescence emission of FAD and FMN as a function of pH. Six nmoles/200 μl protein sample was boiled for 3 min in the dark, centrifuged at 14,000 g for 10 min and rapidly cooled on ice. Then 6 μl of the clear supernatant were analyzed as described by the method of Faeder and Siegel [25]. The 6μl were diluted with standard buffer (0.1 M potassium phosphate buffer pH 7.7, containing 0.1 mM EDTA) so that the final volume was 1.5 ml and transferred to a 1-cm path-length fluorimeter cuvette. Fluorescence emission was recorded at 535 nm; the excitation wavelength was 450 nm. After the initial fluorescence measurement at pH 7.7, the pH of the solution was adjusted to 2.6 by the addition of 0.1 ml of 1 N HCl and the fluorescence determined again.

Determination of FAD and FMN dissociation constants

The binding affinity of FAD or FMN on P450 3A4-apoBMR was measured fluorimetrically (Perkin-Elmer LS 55 fluorimeter) with excitation at 450 nm and emission at 535 nm. The Kd of FAD was determined as follow: the intensity of the FAD fluorescence emission was measured after successive small additions (1-2 μl) of 0.6 mM FAD to 1 ml potassium phosphate buffer 0.1M pH 8.0, containing 0.1 mM EDTA and 180 pmoles P450 3A4-apoBMR. The increasing fluorescence emission values (F) (corrected for the blank where only FAD was omitted) were plotted in a double reciprocal graph of inverse fluorescence values as a function of the inverse of FAD concentrations. The Kd of FMN was determined as follow: FMN fluorescence was measured after successive small additions (1-2 μl) of 30 μM P450 3A4-apoBMR to 1 ml 0.1M potassium phosphate buffer pH 8.0, containing 0.1 mM EDTA and 300 pmoles FMN. The difference (ΔF)

from fluorescence values and the value of the blank, where only P450 3A4-apoBMR was omitted, were plotted in a double reciprocal graph of inverse ΔF values as a function of the inverse of P450 3A4-apoBMR concentrations and the -1/Kd values were determined from the X-intercept.

Activity of apoBMR-domain as a function of flavins

The NADPH oxidation activity of the apo-BMR domain was investigated spectrophotometrically studying the rate of decrease in absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) of the reduced species varying flavins concentration. Reaction mixtures were performed in an open 1 cm pathway quartz cuvette and at room temperature: 150 nmoles of NADPH, the exact concentration of which was previously determined spectrophotometrically at 340 nm, were added to 1 ml final volume of 0.1 M potassium phosphate buffer at pH 8.0, containing 5 mM $MgCl_2$, 100 mM KCl and 200 pmoles P450 3A4-apoBMR and appropriate equimolar amounts of each of the flavin solutions in the 0-15 μM range. The NADPH absorbance was recorded during a reaction time of 3 minutes changing FAD and FMN concentration from 0 to 15 μM. The same experiments performed in the presence of 20 μM erythromycin gave no differences in the reaction rate.

Effect of exogenous flavins on the reconstituted apo-protein activity was clearly highlighted by plotting the NADPH oxidation rate (ΔA/min) as a function of flavins concentration.

Activity of flavin-reconstituted P450 3A4-BMR

Enzymatic activity was measured by monitoring the erythromycin N-demethylation reaction in saturating condition of all substrates and cofactors. The reaction mixture contained in 1 ml final volume of 0.1 M potassium phosphate buffer pH 8.0, containing 5 mM $MgCl_2$, 100 mM KCl, 120 nmoles of NADPH (exactly determined spectrophotometrically at 340 nm), 15 μM FAD and FMN, 160 pmoles P450 3A4-apoBMR and appropriate amounts of erythromycin in the range 0-25 μM. Zero-time controls were performed for all experiments by omitting substrate. The reaction mixtures were incubated for 10 min at 37°C after which the reaction was stopped by the addition of 50 μl 37% hydrochloric acid. The quantity of formaldehyde produced was then determined in a 0.5 ml aliquot as described below.

Study on coupling efficiency

Reactions were carried out at 37°C in a 1 cm pathway quartz cuvette and consisted of the following mixture: 1.25 ml 0.1 M potassium phosphate buffer pH 8.0, 2% glycerol, 5 mM $MgCl_2$, 100 mM KCl, 200 pmoles of P450 3A4-apoBMR, 15 μM FAD and FMN and varying concentrations of erythromycin (0 to 25 μM).

The absorbance was blanked and 150 nmoles of NADPH was added and rapidly mixed; reaction was followed at 340 nm until absorbance became constant around 0.150-0.200. Then two aliquots of 0.5 ml were drawn in separated tubes and the reaction was terminated by the addition of 50 μl of 37% hydrochloric acid for formaldehyde determination and 40 μl of 70% perchloric acid for hydrogen peroxide determination, whilst placed on ice. Appropriate controls were performed by omitting only NADPH from the reaction mixture. The possible formation of superoxide anion

during the demethylation reaction was measured on incubations performed in the same conditions but in the presence of 200 units of superoxide dismutase (SOD).

The % coupling efficiency was calculated as: HCHO produced / NADPH oxidized x 100.

Formaldehyde determination

Formaldehyde resulting from erythromycin N-demethylation was measured using the NASH reagent [26] as described by Dodhia et al. [7] with minor modifications: 0.5 ml of terminated reaction mixture was vortexed and centrifuged at 13,000 x g for 5 minutes and 0.5 ml of the clear supernatant was transferred to another tube and vigorously mixed with 0.5 ml of freshly prepared NASH reagent (3 g ammonium acetate, 40 μl acetylacetone, 60 μl acetic acid in 10 ml of water). The mixture was incubated at 50°C for 30 min in the dark and, after cooling at room temperature, the absorbance at 412 nm was measured. In these conditions the rate of N-demethylation of erythromycin was linear up to 30 min. A calibration curve was constructed by incubating 0.5 ml potassium phosphate buffer pH 8.0, containing 2% glycerol, 5 mM $MgCl_2$, 100 mM KCl, 15 μM FAD and FMN, 150 nmoles NADPH and a range of formaldehyde concentrations (0-30 μM) with 0.5 ml NASH reagent for 30 min at 50°C.

Determination of hydrogen peroxide

The hydrogen peroxide was determined using the colorimetric method described by Sugiura et al. [27]. To 0.5 ml of reaction mixture (0.1 M potassium phosphate buffer pH 8.0, 2% glycerol, 5 mM $MgCl_2$, 100 mM KCl, 80 pmoles of P450 3A4-apoBMR, 15 μM FAD and FMN, erythromycin varying from 0 to 25 μM and 150 nmoles NADPH) 4 N KOH was added until the pH was

neutralized. In order to remove the protein the mixture was centrifuged as before. To the clear supernatant 0.5 ml of reactive reagent (consisting of 50 ml potassium phosphate buffer pH 8.0, 20 μl N,N-dimethylaniline, 8 mg 4-amino-2,3dimethyl-1-phenyl-3-pyrazolin-5-one and 300 units of crude horseradish peroxidase) was added and gently vortexed. After 30 seconds absorbance at 550 nm was measured and compared with a calibration curve constructed by incubating standards solutions containing a range of freshly prepared hydrogen peroxide concentrations from 0 to 200 μM in a final volume of 0.5 ml of the same buffer previously described.

Results and discussion

Reductase requirements for the electron transfer chain

In physiological systems, electrons are transferred from NADPH to the FAD and then to the FMN within the reductase and from here to the heme iron in the P450 domain. In order to confirm that the electrons flow through the different redox centres in the same way in the P450 3A4-BMR chimera, the apo-form lacking the flavin cofactors was prepared and the NADPH consumption was measured upon their individual or combined additions. Strategies to remove flavins from proteins include lowering the pH, increasing the salt concentration, changing the solvent and increasing the temperature. As the final aim of our study was to obtain highly re-constitutable apoprotein, the milder process of varying the salt concentration was chosen and the P450 3A4-apoBMR was prepared by treatment of the native holo-enzyme with 200mM concentration of KCl. Fluorimetric data confirmed that after treatment of the purified fusion-protein with potassium chloride it was depleted in its flavin content with a FAD/protein ratio of 8.9×10^{-3} and FMN/protein of 23.6×10^{-3}. Analysis of the heme content in the P450 3A4-apoBMR fusion protein showed that the salt treatment had a negligible effect on the heme content and that the fusion protein contained 0.92 ± 0.02 mol of heme per mol of fusion protein (mean ± SD of 4 separate measurements) [28] Spectrophotometric analysis of the reduced P450 3A4-BMR in the presence of carbon monoxide showed the characteristic shift of the Soret peak from 419 to 450 nm, with the appearance of a negligible shoulder at 420 nm [29].

Once obtained the P450 3A4-apoBMR, the ability of the protein to re-incorporate the two flavin cofactors was investigated by fluorimetric titrations. The binding of both FAD and FMN to P450 3A4-apoBMR was found to follow a hyperbolic trend and the resulting dissociation constants were determined to be 4.8×10^{-6} M and 5.2×10^{-7} M for FAD and FMN, respectively.

The next step was to measure NADPH oxidation by P450 3A4-apoBMR at increasing concentration of flavins (Figure 1). As the electrons are transferred from NADPH to FAD, FMN and then heme, the higher final values of activity achieved by addition of FAD compared to those obtained for the FMN is consistent with the 2.65 fold higher initial residual concentration of FMN content in the apo-BMR. Moreover, kinetics data highlight that when FAD and FMN are added together in equimolar concentrations, the NADPH oxidation rate observed is faster than that in the presence of the separate cofactors. In particular the apo-protein was saturated by a flavin concentration of 15 μM. These results demonstrate that the electrons flow from NADPH to FAD to FMN, as in the human CPR.

The NADPH oxidation experiments on the P450 3A4-apoBMR were also performed in the presence of erythromycin as the substrate but no significant differences were observed. This suggested that regardless of the presence or absence of substrate the majority of the reducing equivalents from NADPH are directed to uncoupled reactions involving oxygen. Furthermore, thermal denaturation experiments showed that NADPH oxidation was completely stopped when the fusion protein was thermally denaturated confirming that the electron flow depends on a correct folding of the holo 3A4-BMR.

Effect of substrate concentration on the activity of the reconstituted P450 3A4-BMR

The catalytic activity of the flavin-reconstituted P450 3A4-BMR was studied in the presence of the substrate erythromycin, Figure 2A. Fitting of the data to the Michaelis-Menten equation led to calculated K_m of 11.7 μM, V_{max} = 0.32 nanomoles of formaldehyde produced per minute by 200 picomoles of reconstituted fusion protein and k_{cat} of 2.7 x 10^{-2} s^{-1}. The activity of the reconstituted fusion protein was markedly inhibited when substrate:enzyme ratio exceeded the value of 125 (data not shown). In order to determine the production of reactive oxygen species due to

uncoupling of the P450 3A4-BMR cycle, the generation of the superoxide anion and hydrogen peroxide using superoxide dismutase and peroxidase were measured [30]. While the production of superoxide anion (measured via the hydrogen peroxide produced in presence of superoxide dismutase) was found to be absent during the erythromycin N-demethylation, the generation of hydrogen peroxide was evident as shown in Figure 2B. Comparing the amount of formaldehyde and hydrogen peroxide produced at increasing concentrations of erythromycin, it is noticeable that hydrogen peroxide decreases while at the same time the formaldehyde increases.

In particular we determined that the highest level of coupling was achieved in saturation conditions of erythromycin and flavins, after a 10 minute reaction at 37° C. Under these conditions, 115 nanomoles of NADPH were oxidized resulting in the production of 7.3 nanomoles of formaldehyde and 90 nanomoles of hydrogen peroxide, with a coupling efficiency of 6.4 % (Table I). This means that a mere 6.4% of the NADPH consumed by the reconstituted P450 3A4-BMR was used in the N-demethylation of erythromycin, while the remainder was largely used in oxygen reduction products. It is interesting to notice that following the reduction of the reconstituted P450 3A4-BMR by NADPH in the absence of erythromycin as substrate, both the direct (dissociation of peroxy-complex of cytochrome) and indirect formation of hydrogen peroxide (from superoxide anion) were observed. This is due to the fact that in the presence of substrate not all of the redox-equivalents of NADPH are used in hydrogen peroxide and/or superoxide anion-radical formation, leading to some of them being also used in the four-electron reduction of oxygen to water. On the other hand, when BMR is linked to its natural bacterial enzyme, the CYP102A1, it forms a highly coupled system and this has been attributed to its fused nature and the redox states and potentials of the heme, FAD and FMN cofactors [31]. However, our data shows that this assumption no longer holds in the fused P450 3A4-BMR, indicating that the simple fusion of the protein domains in a single polypeptidic chain is not the factor in

preventing uncoupling. Other parameters must be involved such as the correct complementarity in the reductase-P450 complex and/or the nature of the heme environment.

Effect of flavins concentration on the activity of the reconstituted P450 3A4-BMR

In order to assess how FAD and FMN affect the coupling of the enzymatic reaction, the amount of formaldehyde and hydrogen peroxide produced during the N-demethylation of erythromycin were measured by varying the concentration of flavin cofactors. Figure 3 shows that increasing of the concentration of exogenous flavins led to an enhancement of the catalytic activity that resulted in higher amounts of formaldehyde accompanied by generation of lower amounts of hydrogen peroxide.

Further studies were carried out to determine the influence of NADPH concentration on coupling of the enzymatic efficiency. Data reported in Figure 4 show the amount of the formaldehyde and hydrogen peroxide produced as a function of NADPH oxidised, indicating that reducing equivalents provided by NADPH are largely wasted in an uncoupled reaction as previously demonstrated by electrochemical studies [8]. In addition the measure of hydrogen peroxide (Figure 4B) proved once again that erythromycin N-demethylation was uncoupled from electron transfer to such an extent that the majority of the electrons that flow from NADPH did not participate in the production of formaldehyde.

Effect of NADPH concentration on the activity of the reconstituted P450 3A4-BMR

It is also interesting to note that while formaldehyde production increased with increasing NADPH concentration reaching a maximum value, the production of hydrogen peroxide increased linearly, further demonstrating the extent of uncoupling. In fact from the data reported in Figure 4(A) and (B) it is possible to calculate that in saturating concentrations of erythromycin and flavin cofactors when 23.6 nmoles of NADPH were oxidized, 2.0 nmoles formaldehyde, and 20.2 nmoles of hydrogen peroxide were produced, with a coupling efficiency of 8.5 %, whereas when 122.2 nmoles NADPH were oxidised, only 6.2 nmoles of formaldehyde and 105.5 nmoles of hydrogen peroxide were produced, with a coupling factor of 5.1 %. Further increases in the amount of NADPH did not improve the production of formaldehyde but only led to an increase in the hydrogen peroxide produced therefore enhancing of the uncoupling process.

Effect of pH and ionic strength on the activity of the reconstituted P450 3A4-BMR

Finally, the effect of pH and ionic strength on the activity and uncoupling process of the reconstituted P450 3A4-BMR were also studied. As reported in Table II, the most efficient activity of the fusion protein was obtained at pH 8.0 where the production of formaldehyde reached a maximum while hydrogen peroxide reached a minimum. For pH values either side of pH 8.0 there was a concomitant decrease in coupling efficiency. Interestingly, at pH 7.0, the uncoupling level was two folds lower than the one at pH 8.0. Moreover, experiments of enzymatic activity carried out at pH 8.0 in saturating amounts of substrate and flavin cofactors, as a function of ionic strength in the range 25-500 mM potassium phosphate, demonstrated that the best reaction conditions were those carried out in 50 mM buffer concentration.

In conclusion, there are several regulatory features to consider for electron transfer chain in P450 cytochromes. FAD and FMN added to apo-enzyme in over-saturating concentrations neither influence formaldehyde production nor coupling efficiency. Conversely, the optimal conditions of

coupling efficiency depend only on the NADPH concentration, i.e. it is clear that an excess of NADPH results in an increase in the amount of hydrogen peroxide produced without additional gain in formaldehyde production, but with a marked decrease in the coupling efficiency. The pH (pH 8.0) and ionic strength (50 mM potassium phosphate) were also found to modulate the level of coupling indicating an influence over the formation of a productive interaction(s) between the BMR and the heme domains. The highest coupling efficiency reached in the engineered P450 3A4-BMR was 6.4 % that is comparable to the native P450 3A4.

Overall the data presented in this paper indicate that uncoupling is an intrinsic property of the heme-domain, and the covalent linkage of the reductase in a single polypeptide chain has little influence over the activity coupled to product formation.

Acknowledgements

This work was supported by the Region Piedmont CIPE 2006 (CYP-TECH project, Italy) and the PRIN 2007 (Engineering Cytochrome P450, Italy).

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Table I - Consumption of NADPH and amount of products formed from the erythromycin N-demethylation and coupling efficiency of reconstituted P450 3A4-BMR with varying amounts of erythromycin concentration.

Erythromycin (μM)	nmoles of NADPH consumed	nmoles of coupling products		nmoles of uncoupling products		% of coupling efficiency[4]
		HCHO[1a]	$H_2O_{pr.}$[1b]	H_2O_2[2]	$H_2O_{unc.}$[3]	
0	123.6	0	0	122.5	1.1	0.0
5	116.2	3.6	3.6	105.9	6.7	3.1
10	118.0	5.4	5.4	102.3	10.3	4.6
15	118.5	6.9	6.9	99.5	12.1	5.8
20	115.0	7.3	7.3	90.0	17.7	6.4

[1a,1b] HCHO and $H_2O_{pr.}$ are produced in the coupled reaction from the N-demethylation.
[2] H_2O_2 arise from two-electron uncoupling.
[3] H_2O_{unc} arise from four-electron oxidase uncoupling and is calculated by the difference between the molecules of NADPH consumed minus the molecules of HCHO and H_2O_2 generated.
[4] The % of coupling efficiency is calculated as: HCHO x 100 / NADPH consumed

Table II – Effect of pH on coupling efficiency of the reconstituted P450 3A4-BMR.

pH	nmoles of NADPH consumed	nmoles of HCHO	nmoles of H_2O_2	% of coupling efficiency
7.0	**142.8**	**4.2**	**101.2**	**2.9**
7.5	**139.6**	**6.0**	**95.9**	**4.3**
8.0	**137.0**	**8.4**	**91.2**	**6.1**
8.5	**138.7**	**6.5**	**99.9**	**4.7**
9.0	**139.2**	**5.4**	**102.9**	**3.9**

Figure Legends

Fig 1: NADPH oxidation activity of apo-BMR domain at different concentrations of FAD (triangle), FMN (circle) and equimolar FAD-FMN (square). 150 nmoles of NADPH in 1 ml final volume of 0.1 M potassium phosphate buffer pH 8.0 containing 5 mM $MgCl_2$, 100 mM KCl and 200 pmoles apo-P450 3A4-BMR were added to different concentration of flavins.

Fig 2: (A) Erythromycin N-demethylase activity measured as HCHO production by reconstituted P450 3A4-BMR as a function of erythromycin concentration and **(B)** hydrogen peroxide production as a function of erythromycin concentration during incubation with reconstituted P450 3A4-BMR. Both reactions were carried out at 37°C for 10 min.

Fig 3: (A) Effect of varying equimolar concentrations of FAD and FMN on the erythromycin N-demethylation activity and **(B)** hydrogen peroxide production by the reconstituted P450 3A4-BMR. 150 nmoles of NADPH in 1.25 ml of 0.1M potassium phosphate buffer pH 8.0 with 2% glycerol, 5 mM $MgCl_2$, 100 mM KCl, FAD and FMN in the range 0-15 μM, 25 μM erythromycin and 200 pmoles of P450 3A4-apoBMR.

Fig 4: **(A)** Formaldehyde produced by the erythromycin N-demethylation and **(B)** the linear relationship between hydrogen peroxide produced by the reconstituted P450 3A4-BMR, both as a function of the concomitant NADPH oxidation. Reaction mixture included 15 μM FAD and FMN, 25 μM erythromycin and 200 pmoles of P450 3A4- apoBMR.

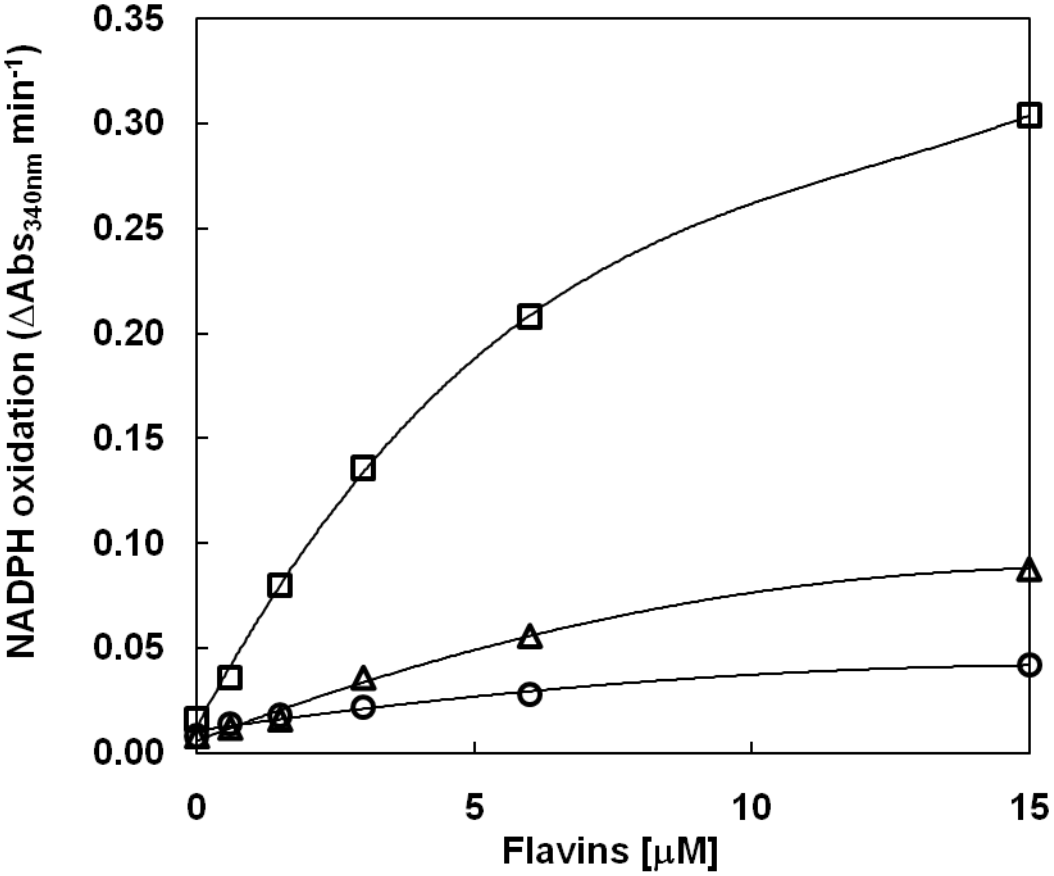
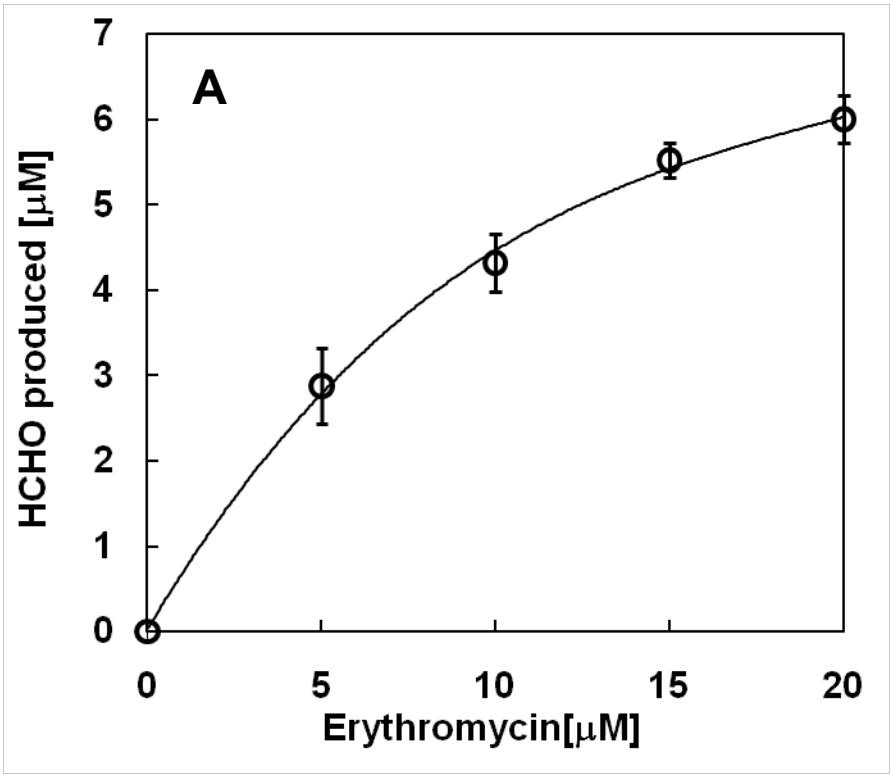

NADPH oxidation (ΔAbs340nm min-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
Flavins [μM]

FIGURE 1


A
HCHO produced [μM]
0
1
2
3
4
5
6
7
0
5
10
15
20
Erythromycin[μM]

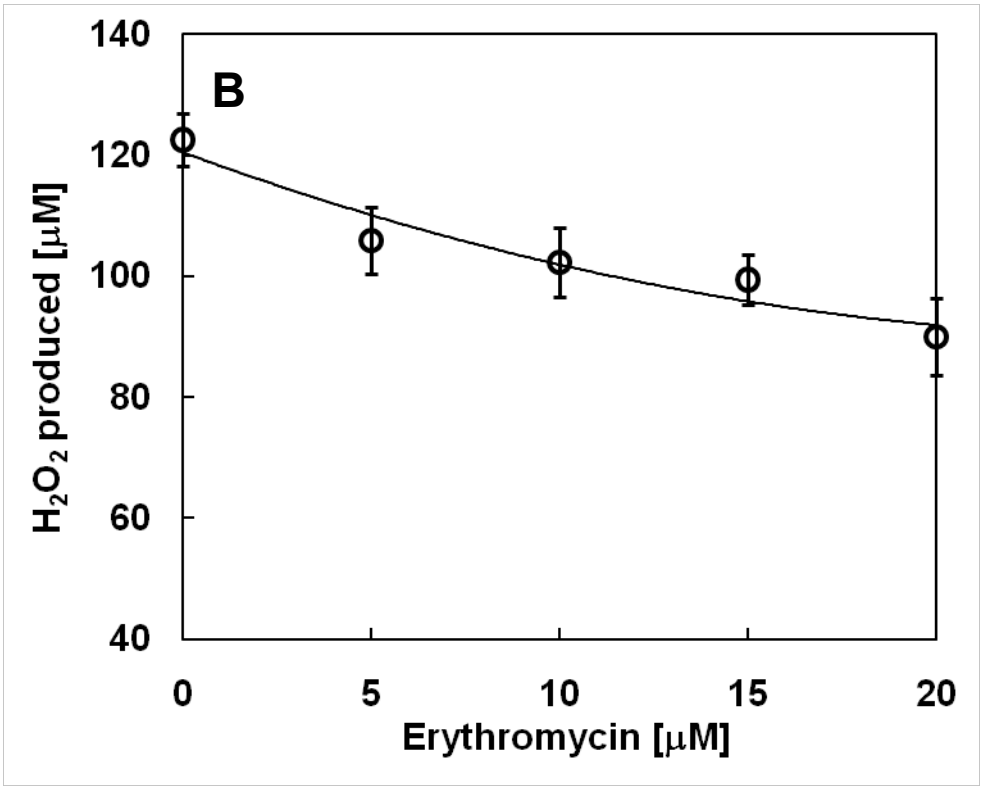
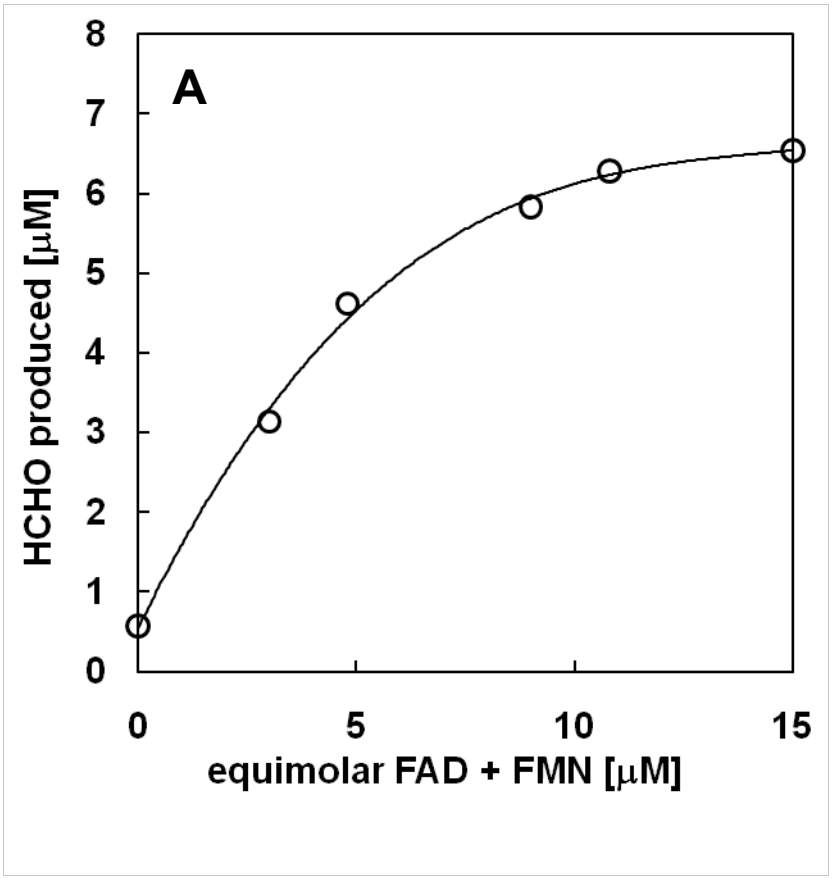

B
H2O2 produced [μM]
40
60
80
100
120
140
0
5
10
15
20
Erythromycin [μM]

FIGURE 2


A
HCHO produced [μM]
0
1
2
3
4
5
6
7
8
0
5
10
15
equimolar FAD + FMN [μM]

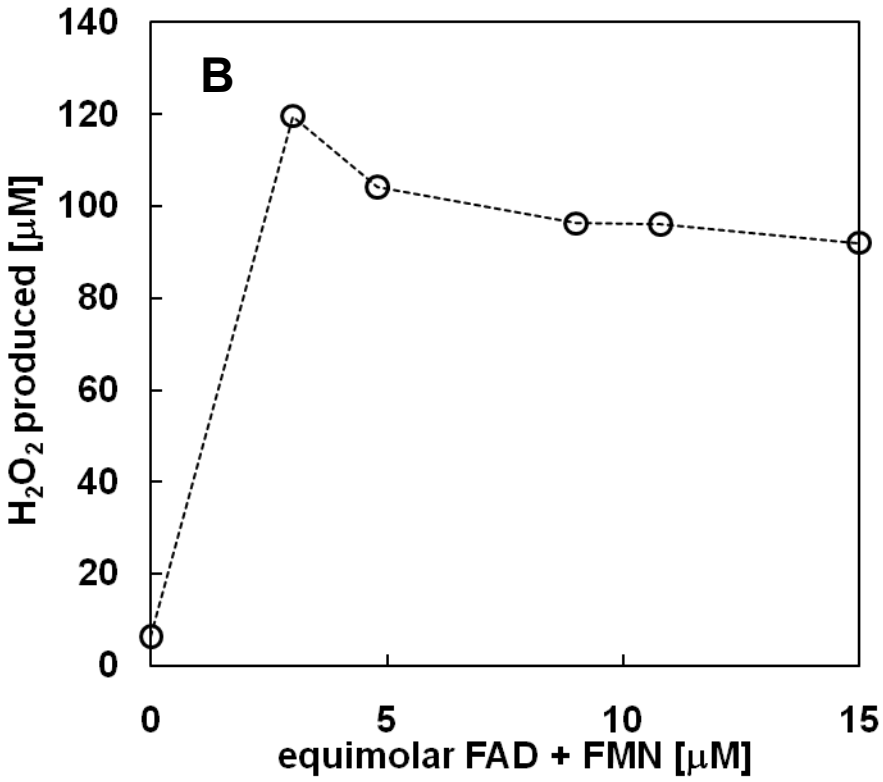
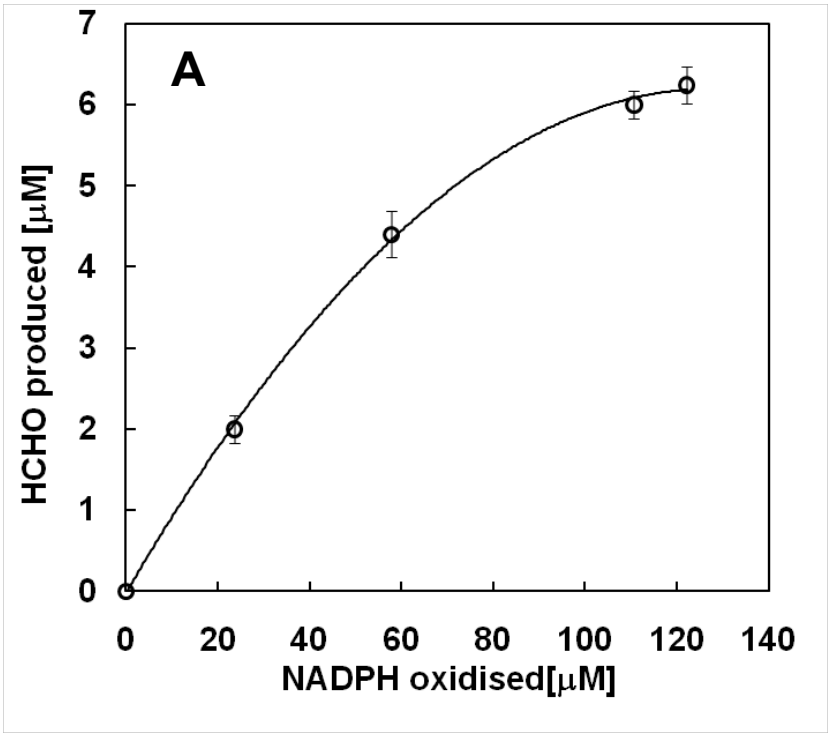

B
H2O2 produced [μM]
0
20
40
60
80
100
120
140
0
5
10
15
equimolar FAD + FMN [μM]

FIGURE 3


A
HCHO produced [μM]
NADPH oxidised[μM]
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140

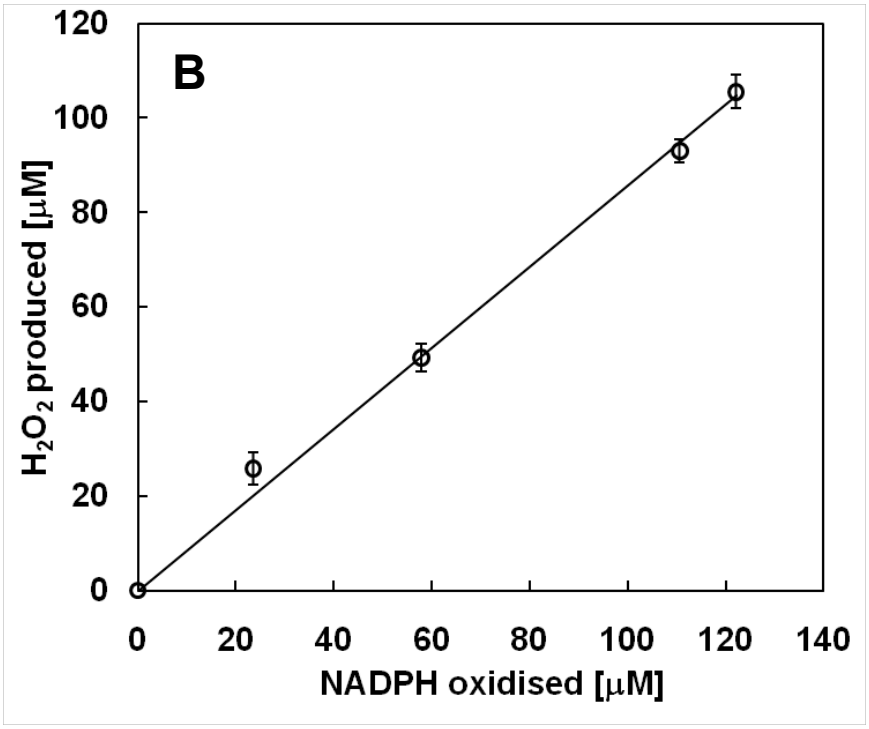

B
H_2O_2 produced [μM]
NADPH oxidised [μM]
0
20
40
60
80
100
120
0
20
40
60
80
100
120
140

FIGURE 4