Bovine and cyanobacterial electron carrier proteins as electron donors to CYP106A2 from *Bacillus megaterium* ATCC 13368

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Abbreviations: ET, electron transfer; *An*Fd, *An*Fd_{ox}, *An*Fd_{rd}, Ferredoxin from the cyanobacterium *Anabaena* PCC 7119 and it is oxidised and reduced states, respectively; *An*FNR, *An*FNR_{ox}, *An*FNR_{sq}, *An*FNR_{hq}, Ferredoxin-$NADP^+$ reductase from the cyanobacterium *Anabaena* PCC 7119 and in its oxidised, semiquinone and hydroquinone states, respectively; boAdx4-108, boAdx4-108_{ox}, boAdx4-108_{rd}, truncated bovine adrenodoxin consisting of residues 4-108 (lacking amino acids 1-3 and 109-128) and in its oxidized and reduced states; *Bm*CYP106A2, *Bm*$CYP106A2_{ox}$, *Bm*$CYP106A2_{rd}$, CYP106A2 from *Bacillus megaterium* ATCC 13368 and in its oxidized and reduced states; boAdR, bovine adrenodoxin reductase; K_d, dissociation constant; $k_{A>B}$, $k_{B>C}$ pseudo first-order observed rate constants; WT, wild-type; Cytc, $Cytc_{ox}$, $Cytc_{rd}$ horse heart cytochrome *c* and in its oxidized and reduced states respectively.

Summary

The CYP450 from *Bacillus megaterium* (*Bm*CYP106A2) catalyzes the 15β-hydroxylation of several steroids and also synthesizes mono-hydroxylated 9α- and 11α-OH-progesterone *Bm*CYP106A2. The present study reports on the ability of *Bm*CYP106A2 to be efficiently reduced by a truncated bovine adrenodoxin mutant, as well as by the photosynthetic flavodoxin and particularly ferredoxin electron carriers from the cyanobacterium *Anabaena*. These results open the possibility for the design of a hybrid system to provide reducing equivalents for the hydroxylation process. Additionally, they suggest that despite the interaction of *Bm*CYP106A2 with these proteins, particularly with flavodoxin, do not rely on a precise complementarity of the reacting molecules, rearrangements might be required and alternative binding modes might contribute to the observed ET reactions.

Introduction

Steroid compounds can be ranked among the most widely marketed products from the pharmaceutical industry. Their manufactured products have a wide range of therapeutic purposes (anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic or contraceptive agents between others) (Fernandes *et al.*, 2003). Their complexity requires complicated multi-step chemical synthesis, which usually evolve with low yields. The use of biocatalysts in their production is particularly interesting because they present high regio- and stereo-selectivity and are environmentally friendly (Straathof *et al.*, 2002). Thus, the use of microorganisms, or their isolated enzymes, provides a cheap and efficient valid alternative to chemical synthesis (Mahato & Garai, 1997; Guengerich, 2002; Fernandes *et al.*, 2003; Manosroi *et al.*, 2003). Steroid converting enzymes are found in mammals, plants, fungi and bacteria (Hannemann *et al.*, 2006). Prokaryotic cytochrome P450s (CYP450s) are soluble, and therefore easy to purify and to handle, but they need to interact with their soluble electron donor partners to display functional activity (McLean *et al.*, 2005). The CYP450 from *Bacillus megaterium* ATCC 13368 (*Bm*CYP106A2) was identified as a few of the bacterial steroid hydroxylating enzymes (Berg *et al.*, 1979a; Berg *et al.*, 1979b). *Bm*CYP106A2 catalyzes the 15β-hydroxylation of several steroids (11-deoxycorticosterone, testosterone, progesterone, and corticosterone) and besides 15β-OH-steroids, also synthesizes mono-hydroxylated 9α- and 11α-OH-progesterone (Lisurek *et al.*, 2004; Hannemann *et al.*, 2006). Both functions have pharmaceutical relevance because 9α-OH-steroid derivatives exhibit glucocorticoidal and progestational activity (Masuda *et al.*, 1987) and, 11α-OH-steroids might exhibit antiandrogenic activity with minimal estrogenic and progestational side effects (Tamm *et al.*, 1982). A key problem preventing the biotechnological use of isolated CYP450s is the *in vitro* electron supply

to their heme cofactor (Urlacher *et al.*, 2004). *In vivo*, NAD(P)H supplies electrons to CYP450s through a considerable diversity of ET chains. The wide diversity of CYP450s electron donors highlights their versatility and adaptability (McLean *et al.*, 2005). NAD(P)H is, however, too expensive to be used with biotechnological purposes. The use of electron mediators (Schwaneberg *et al.*, 2000), direct electron supply from electrodes (Bes *et al.*, 1995; Kazlauskaite *et al.*, 1996; Madoz-Gúrpide *et al.*, 2000), as well as enzymatic (Fernandez-Salguero *et al.*, 1993; Taylor *et al.*, 2000; Vincent *et al.*, 2007), and organometallic systems (Hollmann *et al.*, 2002) might be also sources of the required reduction equivalents, which coupled to hybrid protein systems might be a solution (Bes *et al.*, 1995).

The biological function and the electron donor of *Bm*CYP106A2 are unknown, but the bovine adrenal adrenodoxin reductase (boAdR)/Adrenodoxin (boAdx) and the *Pseudomonas putida* putidaredoxin reductase/putidaredoxin systems, as well as the ET chain from *Bacillus subtilis* are able to supply *Bm*CYP106A2 with the reducing equivalents necessary for steroid hydroxylation (Berg *et al.*, 1979a; Berg *et al.*, 1979b; Lambeth *et al.*, 1979; Rauschenbach *et al.*, 1993; Simgen *et al.*, 2000; Lisurek *et al.*, 2004; Agematu *et al.*, 2006; Hannemann *et al.*, 2006; Virus *et al.*, 2006). boAdx and boAdR are structurally and functionally similar to the cyanobacterium *Anabaena* 7119 ferredoxin (*An*Fd) and ferredoxin-$NADP^+$ reductase (*An*FNR), which lead to NADPH production during photosynthesis (Hurley *et al.*, 2002). In this later process the *An*Fd function can be also replaced by a flavoprotein, flavodoxin (*An*Fld) (Medina & Gomez-Moreno, 2004). Hybrid systems from these two ET chains have been studied, but heterologous *An*FNR/boAdx, boAdR/*An*Fd and boAdR/*An*Fld chains, despite allowing ET and CYP450s reduction, were considerably less efficient than the physiological ones in reducing boCYP11A1 (Faro *et al.*, 2003; Zollner *et al.*, 2004).

Here, we report on the ability of bovine adrenal and photosynthetic ET chains to efficiently reduce *Bm*CYP106A2. Optical biosensor, isothermal titration calorimetry (ITC) and steady-state and pre-steady state kinetics methods have been used to characterize the efficiency of these processes. Our data indicate that *Bm*CYP106A2 is able to accept electrons from boAdx4-108, *An*Fd and *An*Fld. The use of different *An*Fld mutants as electron donor to *Bm*CYP106A2 indicates that changes in redox properties of the ET protein influence the overall efficiency of the process.

Materials and Methods

Biological material.

Recombinant WT FNR, WT Fd and WT and mutated Fld proteins from the cyanobaterium *Anabaena* sp. PCC7119 were overexpressed and purified from *Luria-Bertani* (LB) cultures of IPTG-induced *Escherichia coli* transformed with the pTrc99a vectors containing the corresponding *Anabaena* genes (Fillat *et al.*, 1991; Hurley *et al.*, 1993; Medina *et al.*, 1998; Casaus *et al.*, 2002; Frago *et al.*, 2007). *Bm*CYP106A2 was expressed in *E. coli* and purified as described elsewhere (Rauschenbach *et al.*, 1993; Simgen *et al.*, 2000; Lisurek *et al.*, 2004). The recombinant boAdR and the truncated form of boAdx (boAdx4-108), presenting a more negative reduction potential than the WT form, were produced following standard protocols (Uhlmann *et al.*, 1992; Sagara *et al.*, 1993). UV-Vis spectra and SDS-PAGE were used as purity criteria.

Spectroscopic analysis

UV/Vis spectra were recorded on a Cary 300 or on a Shimadzu UV2100 spectrophotometer. Extinction coefficients used for quantification were ε_{458} = 9.4 $mM^{-1}cm^{-1}$ for WT *An*FNR (Pueyo & Gómez-Moreno, 1991), ε_{422} =9.7 $mM^{-1}cm^{-1}$ for WT *An*Fd, those reported for the different *An*Fld forms used in the present work (Lostao *et al.*, 1997; Frago *et al.*, 2007), ε_{450} = 11.3 $mM^{-1}cm^{-1}$ for boAdR (Chu & Kimura, 1973; Hiwatashi *et al.*, 1976) and ε_{414} = 9.8 $mM^{-1}cm^{-1}$ for boAdx4-108 (Huang & Kimura, 1973). The *Bm*CYP106A2 concentration was measured by CO difference spectra. Blank measurements were performed by recording the absorbance at 450 nm and 490 nm of the dithionite-reduced samples; then the samples were saturated with CO by bubbling the gas for 1 min and the absorbance values at 450 nm and 490 nm recorded. The *Bm*CYP106A2 concentration was calculated by using $\varepsilon_{450\text{-}490}$ = 91 $mM^{-1}cm^{-1}$ (Omura & Sato, 1964).

Cross-linking experiments

Cross-linking reactions were performed at 25 °C using 30 µM of each protein and 2 mM 1-ethyl-3-[(3-dimethylamino) propyl)-carbodiimide (EDC). The reaction was stopped after 1h by Laemmli-buffer addition (Laemmli, 1970), followed by separation of the proteins by SDS-PAGE. *An*Fd has a molecular mass of 11 kDa, *An*Fld of 18.8 kDa (Fillat *et al.*, 1991) and *Bm*CYP106A2 of 47.5 kDa (Virus *et al.*, 2006).

Optical biosensor measurements

Binding between *Bm*CYP106A2 and boAdx4-108, *An*Fd or *An*Fld was assayed using a BIAcore 3000 system and the optical biosensor method previously described with slight modifications (Zollner *et al.*, 2008). Throughout the measurement a continuous flow of BIAcore HEPES buffer (HBS-P) pH 7.4 and EDTA 50 µM was maintained over the sensor surface. Immobilization of His-tagged *Bm*CYP106A2 (*Bm*CYP106A2-HIS) (R= 1.76) 0.3 µM to a NTA sensor chip was performed as previously described (Nieba 1997). Binding of the protein carrier to *Bm*CYP106A2 was analyzed after injection of protein solutions with varying concentrations in the range 10 -500 µM. Determination of K_d values was achieved by using the BIA evaluation software 4.1.

Enzymatic activity

The efficiency of the *Bm*CYP106A2-dependent deoxicorticosterone (DOC) conversion to 15β-hydroxydoxicorticosterona (15β-OH-DOC) when receiving electrons from boAdR or *An*FNR via the different electron carriers, boAdx4-108, WT *An*Fd, WT and mutated *An*Flds, was assayed by an adapted technique of that described previously (Lisurek *et al.*, 2004; Virus *et al.*, 2006). The 15β-hydroxilase activity of the *Bm*CYP106A2 was measured in a final volume of 500 µL at 30 °C, for 3 min, in 50 mM HEPES, pH 7.4 and in the presence of a NADPH regenerating system. The

concentrations used were; 0.5 µM *An*FNR or boAdR; a 0.1 - 25 µM range for boAdx4-108 or *An*Fd; 1 - 120 µM range for the *An*Fld variants; 0.25 µM *Bm*CYP106A2; a NADPH-generating system (consisting in $MgCl_2$ 1 mM, glucose-6-phosphate 5 mM, glucose-6-phosphate-dehydrogenase 2.8 UI/ml, and NADPH 0.1 mM); and 400 µM DOC as substrate. The reaction was stopped and the steroids were twice-extracted with an equal volume of chloroform. The organic phase was evaporated and the steroids resolved in 200 µl acetonitrile and, separate on a Jasco HSS1500 reverse-phase HPLC system (with a 3.9 x 150 mm Waters Nova Pak Nukleosil C18 column using as mobile phase 60:40 acetonitrile:destilled water, at 1.0 ml/min and 25 °C). The absorbance was measured at 240 nm. 75 µM of corticosterone in methanol was used as steroid internal standard (Std). The relative amount of the product (P) was determined using the relative peak area of the Std and, the hydroxylation activity was calculated using the equation: $[P] = [Std] \times AUC_P / AUC_{Std}$, were AUC is the area under curve. Each reaction was performed a minimum of three times. Data were fit to the Michaelis-Menten equation to obtain k_{cat} and K_m parameters of each reaction. Errors in the estimated values were ± 10 % and ± 15 %, respectively.

Stopped-flow kinetic measurements.

Stopped-flow experiments were carried out under anaerobic conditions using an Applied Photophysics SX18.MV-R interfaced with a photodiode array detector (300 - 1100 nm) with a minimum integration time of 1.28 ms. All reactions were carried out in 50 mM HEPES pH 7.4, containing 0.05 % Tween20 and at 25°C. Anaerobic boAdR:boAdx4-108, *An*FNR:*An*Fd or *An*FNR:*An*Fld 1:10 mixtures were reduced by the addition of 200 µM anaerobic NADPH; with this method *An*Fld is only reduced to the semiquinone state (Medina & Gomez-Moreno, 2004). Reduced ET proteins were mixed in the stopped-flow with *Bm*CYP106A2 previously incubated with CO under

anaerobic conditions. Reactions were followed at 450 nm, where a pronounced peak is formed upon formation of the ferrous-carbon monoxide CYP450 complexes (Zollner *et al.*, 2002). The proteins were mixed at different molar ratios, keeping active *Bm*CYP106A2 at 1 μM (final concentrations are indicated), and increasing ET protein concentrations: boAdx4-108 and *An*Fd from 1 μM to 30 μM, and *An*Fld concentrations from 2.5 μM to 20 μM. Time-dependent spectral deconvolution was performed by global analysis and numerical integration methods (Pro-K, App. Photo.). Data were fit to multiple step (A<>B>C or A<>B'>C') models, to determine the apparent conversion rate constants ($k_{A>B}$, $k_{B>C}$). Errors in their estimated values were ± 25%.

High-sensitivity isothermal titration calorimetry (ITC) experiments.

ITC measurements were carried out using a high precision VP-ITC calorimetric system (MicroCal LLC, Northampton, MA). The *Bm*CYP106A2 solution (12.04 μM of active cytochrome) in the calorimetric cell was titrated with boAdx4-108 (136.6 μM), *An*Fd (181.9 μM) or *An*Fld (184 and 463.8 μM) dissolved in the same buffer (potassium phosphate 20 mM, pH 7.4, 0.05 % Tween20 and 50 to 100 μM cholestenone). All solutions were properly degassed and carefully loaded into the cells to avoid bubble formation during stirring. The experiments were carried out at 25 ºC and with a constant stirring of 307 rpm. The heat evolved after each ligand injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution, the latter estimated as a constant heat throughout the experiment and included as an adjustable parameter in the analysis.

Results

Analysis of cross-linked complexes

Incubation of *Bm*CYP106A2 with either *An*Fd (Fig 1, band 7) or *An*Fld (Fig 1, band 8) in the presence of EDC produced the appearance of protein:protein covalent complexes which molecular weights corresponded to *Bm*CYP106A2:*An*Fd and *Bm*CYP106A2:*An*Fld interactions with 1:1 stoichiometries. Figure 1 also indicates that stoichiometries 1:2 might not be discarded for complexes with both proteins.

Biacore binding data

Biacore measurements were performed to investigate the binding of the different proteins (boAdx4-108$_{ox}$, *An*Fd$_{ox}$ or *An*Fld$_{ox}$) to *Bm*CYP106A2. Only measurements with boAdx4-108 produced sensorgrams compatible with binding (Figure 2), suggesting fast association as well as fast dissociation. However, the decrease of the baseline, attributed to *Bm*CYP106A2 release from the chip, prevented determination of the interaction parameters.

Thermodynamic analysis of boAdx4-108, AnFd and AnFld binding to BmCYP106A2

The thermodynamic parameters for the interaction of *Bm*CYP106A2$_{ox}$ with boAdx4-108$_{ox}$, *An*Fd$_{ox}$ and *An*Fld$_{ox}$ were analyzed by ITC, at 25ºC, in potassium phosphate 20 mM, pH 7.4, 0.05 % Tween20 and 50 - 100 μM cholestenone (Figure 3, Table 1). *Bm*CYP106A2 was able to strongly bind boAdx4-108 with a K_d of 0.08 μM at a single binding site. Similarly, *Bm*CYP106A2 showed a single binding site for *An*Fd, but binding resulted more than 40-fold weaker than that of boAdx4-108 (Table 1). Finally, two binding sites on *Bm*CYP106A2 were detected for *An*Fld, being the averaged interaction 1800-fold weaker than that of boAdx4-108. Direct ITC titrations also allowed determination of the enthalpy and entropy components of the produced interactions. A favorable negative binding enthalpy drove the interaction of

*Bm*CYP106A2 with all these proteins (Figure 4), with an unfavorable entropic contribution of the binding.

*Steady-state kinetic parameters for Bm*CYP106A2 *when using boAdx4-108$_{rd}$, AnFd$_{rd}$ and AnFld$_{sq}$ as electron donors.*

To determine the *Bm*CYP106A2 ability to accept electrons from boAdx4-108, as well as from the photosynthetic electron carrier proteins *An*Fd and *An*Fld, its hydroxylase activity in the conversion of DOC into 15β-OH-DOC was assayed using different electron donor systems. When electrons were provided to *Bm*CYP106A2 through boAdx4-108 previously reduced by a system consisting of NADPH as primary electron donor and the boAdx4-108 physiological donor, boAdR, the reaction took place with a k_{cat} of 216 min^{-1} and a $K_m^{boAdx4\text{-}108}$ of 4.2 μM, indicating that the bovine AdR/Adx4-108 ET chain can interact and donate electrons to the *Bm*CYP106A2. However, when boAdx4-108 was replaced by the photosynthetic *An*Fld, no hydroxylase activity was observed. Such behavior can be related with the low efficiency described for *An*Fld in accepting electrons from boAdR due to the lack of specificity at the interaction surface (Zollner *et al.*, 2004).

Therefore, a second system in which the electron carrier proteins were reduced by NADPH and *An*FNR was used. This system efficiently reduced *An*Fd$_{ox}$ to *An*Fd$_{rd}$ and *An*Fld$_{ox}$ to *An*Fld$_{sq}$ (Medina & Gomez-Moreno, 2004). Additionally, although with considerably less efficiency, it has also been reported to reduce boAdx4-108 (Faro *et al.*, 2003). When electrons were provided to *Bm*CYP106A2 through the hybrid NADPH/FNR/boAdx4-108 system, DOC hydroxylation took place with a k_{cat} considerably slower than if provided by the NADPH/AdR one (8.1 min^{-1} *vs* 216 min^{-1}) but with a similar $K_m^{boAdx4\text{-}108}$ value, 3.1 μM. Therefore, the production of the reduced boAdx4-108 was the rate limiting step (Faro *et al.*, 2003). Noticeably, when the

electrons where provided to *Bm*CYP106A2 by *An*Fd_{rd}, efficiently reduced by its natural *An*FNR partner, CYP106A2 was able to hydoxylate DOC with a turnover (k_{cat} 338 min^{-1}) considerably high (even above the one described when electrons are efficiently donated by boAdx4-108) (Table 2). Additionally, the K_m^{AnFd} (7.34 μM) suggests an interaction of *Bm*CYP106A2 with *An*Fd slightly weaker than that with boAdx4-108. Finally, the NADPH/*An*FNR/*An*Fld system also showed that *An*Fld_{sq} was able to reduce *Bm*CYP106A2. The slower k_{cat} value for the hydroxylation process suggests that *An*Fld_{sq} is less efficient than *An*Fd_{rd} and boAdx4-108_{rd} in donating electrons to *Bm*CYP106A2.(Tables 1 and 2).

Pre-steady-state kinetic analysis of the ET from boAdx4-108_{rd}*, AnFd*$_{rd}$ *and AnFld*$_{sq}$ *to BmCYP106A2.*

Spectral evolution for the reaction of both boAdx4-108_{rd} and *An*Fd_{rd} with *Bm*CYP106A2-CO showed the appearance of the characteristic band at 450 nm typical of a reduced *Bm*CYP106A2-CO interaction, indicating ET from the reduced electron carrier protein to *Bm*CYP106A2 has taken place (Figure 5). At least three species are found along these reactions and the simplest model describing both of them (A<>B>C, B>C corresponding to the appearance of the 450 peak indicating CYP reduction) suggests a slow complex formation-complex rearrangement step ($k_{A>B}$ ~3.6-6 min^{-1}) to reach to the species leading to ET. The ET steps were also slow ($k_{B>C,red}$ ~1.5 min^{-1}), but amplitudes indicated almost full reduction of *Bm*CYP106A2 (Figures 5A and 5B). Rate constants for both processes were apparently independent on the electron donor concentration in the assayed range.

Reaction of *An*Fld_{sq}, generated by the NADPH/*An*FNR reducing system, with *Bm*CYP106A2-CO also showed the appearance of the characteristic band at 450 nm typical of a reduced *Bm*CYP106A2-CO (Figure 5C). Singular value decomposition

analysis suggests that processes with *An*Fld$_{sq}$ were more complicated than with the iron sulfur proteins. In fact, this analysis suggest two supperinpossed processes, A<>B<C and A<>B'>C', and two different complexes (produced in different time scales) appear to account for the two ET steps observed (Figure 5C, inset). Additionally, the rate constants related with *Bm*CYP106A2-CO reduction are one-two orders of magnitude slower than for the other two electron carrier proteins and slightly dependent on the concentration (0.03-0.1 min^{-1} in the 2.5-20 μM rage of Fld).

Steady-state kinetic parameters for BmCYP106A2 when using different AnFld$_{sq}$ variants as electron donors

The 15β-hydroxylase activity of *Bm*CYP106A2 was also assayed using different *An*Fld mutants as electron carrier from NADPH/*An*FNR to *Bm*CYP106A2 to investigate whether *An*Fld variants with different midpoint potentials or properties in their proteins surface modulate the Fld ability to provide electrons to *Bm*CYP106A2 (Table 2). In general, the turnover of *Bm*CYP106A2 resulted considerably diminished when assaying the Fld mutants. However, this number decreased to less than 10 % when replacing the W57 side-chain (stacking the flavin ring at the *re*-face), while considerably smaller effects were detected when using *An*Flds modified at Y94 (stacking the *si*-face) or when simultaneously replacing the only two hydrophobic side chains around the FMN surface in Fld, I59 and I92 (Table 2). With regard to *Bm*CYP106A2 K_m^{Fld}, it was not affected when using I59E/I92E *An*Fld$_{sq}$ as electron donor, but the rest of the introduced mutations slightly decreased it (2- to 8-fold), suggesting, therefore, slightly weaker interactions. Combination of both effects indicated that replacement of W57 in *An*Fld considerably hindered the *Bm*CYP106A2 catalytic efficiency (up to 15-fold when using W57E *An*Fld), while slightly more efficient reactions were observed when using I59A/I92A and Y94A *An*Flds as mediators (Table 2).

Discussion

Thermodynamic parameters for the interaction between boAdx4-108 and *Bm*CYP106A2 suggest a strong interaction between these proteins (Table 1, Figure 3). Steady-state kinetic parameters indicate that this hybrid system, in which a bovine protein provides electrons to a bacterium CYP450, is efficient in the deoxicorticosterone 15β-hydroxylase activity, showing similar rates to those supported by other CYP450s (Zollner *et al.*, 2002; Zollner *et al.*, 2008). This efficiency is considerably reduced if boAdx4-108 gets reduced by *An*FNR (Table 2), in agreement with previous studies indicating that stated the low efficiency of *An*FNR in transferring electrons to boAdx4-108 (Faro *et al.*, 2003). Calorimetric measurements indicate that *Bm*CYP106A2 is also able to interact with *An*Fd and *An*Fld, despite these interactions, particularly with *An*Fld, are considerably weaker than with boAdx4-108. Moreover, *An*Fld can apparently interact at two binding sites on the *Bm*CYP106A2 surface (Table 1), suggesting a lower binding specificity. In all cases (boAdx4-108, *An*Fd, and *An*Fld) the binding to *Bm*CYP106A2 is enthalpically driven, accompanied by an unfavorable entropic contribution. Although the buffer composition makes difficult the direct interpretation of the binding thermodynamic parameters, the unfavorable entropic contribution suggests low desolvation upon binding, as expected in transient protein-protein interactions in electron transfer processes. Compared to boAdx4-108 binding, the affinity reduction for *An*Fd binding is due to a more unfavorable binding entropy ($-T\Delta\Delta S$ = 2 kcal/mol), whereas the affinity reduction for *An*Fld is due to a less favorable binding enthalpy ($\Delta\Delta H$ = 4.6 kcal/mol).

The *Bm*CYP106A2 steady-state parameters when using the NADPH/*An*FNR/*An*Fd system as electron donor are as efficient as those when using the bovine system, but $An\text{Fld}_{sq}$ is 20-fold less efficient. Such decrease is mainly due the

larger K_m^{Fld}, as consequence of the weak interaction of *An*Fld with *Bm*CYP106A2 (Table1). A decrease in the turnover rate is also observed, but it is worth to note that it is just slight having into account that under our experimental conditions *An*Fld_{sq} is the electron donor, with a midpoint potential for the $E_{ox/sq}$ couple (-266 mV) considerably less negative than the $E_{ox/rd}$ of *An*Fd and boAdx4-108 (Table 2) (Hurley *et al.*, 1997; Uhlmann *et al.*, 1997; Nogués *et al.*, 2004). Therefore, the systems including the plant-type and vertebrate-type [2Fe-2S] proteins are similarly efficient in donating electrons to *Bm*CYP106A2, while the flavoprotein is less efficient.

*An*Fd and boAdx4-108 show low sequence identity, but they have conserved topologies in their hydrophobic core domains and [2Fe-2S] interactions, similar charge distribution and dipole moment orientation, suggesting similar docking mechanisms to their electrons donors/acceptors (Bruschi & Guerlesquin, 1988; Hurley *et al.*, 1993; Holden *et al.*, 1994; Uhlmann *et al.*, 1994; Vickery, 1997; Muller *et al.*, 1998; Morales *et al.*, 1999; Muller *et al.*, 1999; Grinberg *et al.*, 2000; Hurley *et al.*, 2000; Hannemann *et al.*, 2001; Muller *et al.*, 2001; Hurley *et al.*, 2002; Zollner *et al.*, 2002; Hurley *et al.*, 2006). The most striking difference between them is related with the [2Fe-2S] cluster accessibility, since the protein shell covering the cluster is slightly thicker in the plant-type Fds. This has been related to their lower redox potential (Muller *et al.*, 1999).

Stopped-flow studies suggest complex rearrangements or conformational gating that might be necessary before an efficient ET can take place. Additionally, the more complex behavior observed with *An*Fld suggests the presence of more than one complex rearrangements leading to ET. Fld shows a strong dipole moment, with its negative end directed towards the flavin ring. The role of this dipole moment in the processes of interaction and ET with positively charged surfaces exhibited by PSI and FNR has been analyzed by introducing single and multiple charge reversal mutations on the Fld

surface. This is in agreement with recent results on the native *An*Fld system that indicate that the interaction with its protein partners do not rely on a precise complementary surface of the reacting molecules. Moreover, these results indicate that the initial orientation driven by the alignment of dipole moment of the Fld molecule with that of the partner contributes to the formation of a bunch of alternative binding modes competent for the efficient ET reaction, being the efficient interactions less specific than in the case of processes with *An*Fd (Medina *et al.*, 2008; Goñi *et al.*, 2009). Comparison of k_{cat} values obtained from the substrate conversion experiments with the maximal observed reduction rates from the stopped-flow experiments indicates that the determined reduction rates of reduced *Bm*CYP106A2–CO complex formation can not enable the steady-state catalytic rates. All together these data indicate a complex behavior and suggest that under our stopped-flow conditions the complexes formed are not the most optimal ones. This is in agreement with previous data reported for human CYP11B1 that indicated more than one site for the redox partner, possibly resulting in the formation of more than one productive complex (Zollner *et al.*, 2008). So far, several models have been used to explain experimental evidences with regard to ET in the mitochondrial CYP450 system (Pikuleva *et al.*, 2000). They include; models in which Adx forms consecutive 1:1 complexes, first with AdR and then with CYP11A1 (Lambeth *et al.*, 1979; Hanukoglu & Jefcoate, 1980; Lambeth *et al.*, 1982); models requiring the formation of an organized 1:1:1 ternary complex between AdR, Adx, and CYP11A1 (Kido & Kimura, 1979; Chashchin *et al.*, 1985; Usanov *et al.*, 1985; Turko *et al.*, 1989); models through 1:2:1 quaternary complexes between AdR, Adx, and CYP11A1 (Hara & Takeshima, 1994); and finally, the possibility that an Adx-CYP450 complex serves as a substrate in accepting electron(s) from an AdR:Adx complex (Light & Orme-Johnson, 1981). Therefore, the ET mechanism might not necessarily be the

same in different mitochondrial CYP450s or under different concentration ratios, especially if hybrid systems as the one here introduced are considered (Beckert & Bernhardt, 1997).

The majority of eukaryotic CYP450S are reduced by an FAD- and FMN-containing NADPH-dependent cytochrome P450 reductase (CPR), getting the electrons directly from the FMN-containing domain evolutionarily related to Flds (Murataliev *et al.*, 2004; Munro *et al.*, 2007). Our data indicate that the photosynthetic *An*Fld can also support *Bm*CYP106A2 reduction (Table 2). Such observation is also in agreement with previous ones: the *Escherichia coli* FldR/Fld system supports the function of bovine P450c17 (Jenkins & Waterman, 1994), flavodoxin cindoxin interacts with CYP176A1 from *Citrobacter braakii* (Hawkes *et al.*, 2002), Flds YkuN and YkuP support the biotin synthesis pathway of CYP107H1 BioI from *B. subtilis* (Lawson *et al.*, 2004). Moreover, P450s:Fld fusion proteins have also been identified (Seth-Smith *et al.*, 2002).

To analyze the steady-state parameters obtained when using different *An*Fld variants we must keep in mind the effect of the introduced mutations on the *An*Fld ability to accept the electron from *An*FNR (Table 3) (Goñi *et al.*, 2008). Replacement of W57 with Glu resulted in a *An*Fld variant less efficient in accepting electrons from FNR than WT Fld, while replacement with Lys or Arg enhanced the *An*Fld ability to accept electrons from *An*FNR (Goñi *et al.*, 2008). Therefore, the decrease in the *Bm*CYP106A2 catalytic efficiency when using W57E Fld might be contributed by this fact, while similar, or more efficient processes, will be expected with the other two mutants. Since this is not the case, particularly for the W57R variant, other parameters must influence the process. The less negative $E_{ox/sq}$ of these *An*Flds surely reduced the driving force of this ET reaction, but changes in the flavin area accessible to the solvent, in the surface electrostatic potential or in the module and orientation of the molecular

dipole reported for these *An*Flds, surely are other factors influencing their ability as electron donors to *Bm*CYP106A2, as reported for the reactions with *An*FNR (Goñi *et al.*, 2008). Despite simultaneous replacement of the two hydrophobic I59 and I92 side chains with Ala or Glu, produced slightly weaker and less efficient interactions with FNR, they were similarly or slightly more efficient in providing electrons to *Bm*CYP106A2 (Tables 2 and 4). This behavior must be related with the mutations making $E_{ox/sq}$ considerably more negative than in WT *An*Fld and, getting it closer to the midpoint reduction potential value exhibited by the best electron donorS here assayed, boAdx4-108 and *An*Fd (Table 2) (Goñi *et al.*, 2008). Additionally, since the positive effect is particularly noticeable in the K_m for the double Ala mutant, other factors must also contribute to the enhanced efficiency. Thus, different shapes at the *An*Fld interactions surface among these variants and WT *An*Fld might be also promoting different orientations and distances between the *An*Fld and *Bm*CYP106A2 redox cofactors, contributing to different binding and ET properties. Finally, replacement of Y94 with Ala has been reported to have a considerably positive effect in the ability of Fld to accept electrons from FNR (Table 3) to produced Fld_{sq} (Casaus *et al.*, 2002). The positive effect in the *Bm*CYP106A2 catalytic efficiency observed when providing electrons with this mutant is due to the stronger interaction between both proteins, since the turnover of the reaction is decreased with regard to WT. The later value is in agreement with the less negative $E_{ox/sq}$ value of Y94A *An*Fld with regard to WT. Therefore, the differential interaction with *Bm*CYP106A2 of this mutant can be related with the removal of the aromatic Tyr side chain that makes the FMN cofactor more accessible to the one-electron reduction process but also influence the protein-protein interaction event**.**

ET between proteins generally requires formation of complexes with a high turnover, characterized by a lifetime of milliseconds and equilibrium dissociation constants in the millimolar to micromolar range (Crowley & Carrondo, 2004). However, rates of ET widely differ between complexes and physiologically they might be related to support the speed of the physiological chain requirements in which they are integrated. Therefore, despite specificity usually contributes to more efficient ET complexes, it might not be such a strong requirement in processes having place in slower time-scales. In this context, the present study reports on the ability of boAdx4-108, *An*Fd and *An*Fld to provide electrons to *Bm*CYP106A2, opening new possibilities for the design of hybrid systems that could make use of self-assembled FNR monolayers coupled to gold electrodes, which will provide the reducing power for the CYP450 hydroxylation processes (Madoz-Gúrpide *et al.*, 2000).

Table 1: Thermodynamic parameters for the binding of *Bm*CYP106A2 to the electron carrier proteins boAdx4-108, *An*Fd and *An*Fld.

	N	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	-TΔS (kcal/mol)
Adx4-108	1	0.08	-9.7	-14.1	4.4
WT *An*Fd	1	3.5	-7.4	-13.8	6.4
WT *An*Fld	2	130	-5.2	-9.5	4.3

Relative error in K_d is 10-15%, whereas absolute error in ΔH and -TΔS is 0.3-0.5 kcal/mol

Table 2: Steady-state kinetic parameters for the deoxicorticosterone 15β-hydroxylase activity of recombinant *Bm*CYP106A2 when using the NADPH/*An*FNR electron donor system and different electron carrier proteins.

	k_{cat} (min^{-1})	K_m[a] (μM)	k_{cat} / K_m[b] ($min^{-1}\mu M^{-1}$)	$E_{ox/sq}$ (mV)	$E_{sq/hq}$ (mV)	$E_{ox/rd}$ (mV)
boAdx4-108	8.2	3.14	2.6			-340[c]
WT *An*Fd	338	7.34	46			-384[d]
WT *An*Fld	80.1	34.2	2.3	-266[e]	-439[e]	
W57E *An*Fld	3.28	20.0	0.16	-255[f]	-419[f]	
W57K *An*Fld	9.51	5.58	1.7	-188[f]	-410[f]	
W57R *An*Fld	5.75	14.3	0.4	-228[f]	-390[f]	
I59A/I92A *An*Fld	43.3	14.4	3	-337[f]	-391[f]	
I59E/I92E *An*Fld	48.9	31.3	1.5	-318[f]	-422[f]	
Y94A *An*Fld	22.6	4.12	5.5	-203[g]	-299[g]	
WT boAdR[b]				-360[h]	-230[h]	-295[i]
WT *An*FNR[c]				-425[j]	-327[j]	-376[j]

[a] K_m for the different electron carrier proteins: $K_m^{boAdx4\text{-}108}$, K_m^{AnFd} or K_m^{AnFld}.

[b] Estimation of enzyme efficiency when [coenzyme] = 0

[c] Data obtained from (Uhlmann *et al.*, 1997)

[d] Data obtained from (Hurley *et al.*, 1997)

[e] Data obtained in 50 mM Tris/HCl at pH 8.0 and 25 °C (Nogués *et al.*, 2004).

[f] Data obtained in 50 mM Tris/HCl at pH 8.0 and 25 °C (Frago *et al.*, 2007).

[g] Data obtained in 50 mM potassium phosphate at pH 7.0 and 25 °C (Lostao *et al.*, 1997).

[h] Data obtained from (Lambeth & Kamin, 1976)

[i] Data obtained from (Lambeth *et al.*, 1976)

[j] Data obtained in 50 mM Tris/HCl at pH 8.0 and 25 °C (Pueyo *et al*, 1991)

Table 3: Relative steady-state kinetic parameters for the *An*FNR NADPH-dependent cytochrome *c* reductase activity and the *Bm*CYP106A2 15β-hydroxylase activity when using different *An*Fld variants as electron carrier proteins.

Mediated by	*An*FNR Cytochrome *c* reductase activity			*Bm*CYP106A2 15β-hydroxylase activity	
	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m^{mut}/K_m^{WT}	$k_{cat}^{mut}/k_{cat}^{WT}$	K_m^{mut}/K_m^{WT}	$k_{cat}^{mut}/k_{cat}^{WT}$
WT	0.7[a]	1[a]	1 [a]	1	1
W57E	0.2[b]	2.1[b]	0.6[b]	0.6	0.04
W57K	1.01[b]	2.7[b]	3.9[b]	0.2	0.1
W57R	0.69[b]	2.3[b]	2.3[b]	0.4	0.1
I59A/I92A	0.45[b]	1.0[b]	0.7[b]	0.4	0.5
I59E/I92E	0.35[b]	1.2[b]	0.6[b]	0.9	0.6
Y94A	12[c]	0.5[c]	5.3 [c]	0.1	0.3

[a] Data from (Medina *et al.*, 1998).

[b] Data from (Goñi *et al.*, 2008).

[c] Data from (Casaus *et al.*, 2002).

Figure Legends

Figure 1. SDS-PAGE for the analysis of the covalent interactions between *Bm*CYP106A2$_{ox}$ and *An*Fd$_{ox}$ or *An*Fld$_{ox}$. (1) MW marker; (2) *Bm*CYP106A2; (3) *An*Fd; (4) *An*Fld; (5) CYP106A2 after EDC treatment; (6) *An*Fld after EDC treatment; (7) mixture of *Bm*CYP106A2 and *An*Fld after EDC treatment and (8) mixture of *Bm*CYP106A2 and *An*Fd after EDC treatment. 1:1 complexes are framed by a white square.

Figure 2. Sensorgram showing the binding kinetics for the interaction of *Bm*CYP106A2$_{ox}$ with boAdx4-108$_{ox}$ (at different concentrations: 10 μM, 50 μM and 200 μM) in real time. The same experiment was repeated in the three channels (continuous line in light grey, grey and black) at the same time. In dotted line is shown the base line (without bounded *Bm*CYP106A2).

Figure 3. Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the titration of (A) *Bm*CYP106A2 with boAdx4-108, (B) *Bm*CYP106A2 with *An*Fd, and (C) *Bm*CYP106A2 with *An*Fld.

Figure 4. Thermodynamic dissection of the interaction of *Bm*CYP106A2 with boAdx4-108, *An*Fd and *An*Fld. The binding Gibbs energy (ΔG), enthalpy (ΔH) and entropy ($-T\Delta S$) are represented in black, dark grey and light grey bars, respectively. Any negative value represents a favorable contribution to the binding, whereas a positive value represents an unfavorable contribution.

Figure 5 Evolution of spectral changes accompanying the reduction of oxidized *Bm*CYP106A2-CO. Time course for the reaction of *Bm*CYP106A2-CO (1μM) with (A), NADPH/boAdR/boAdx (boAdx, 3 μM), (B), NADPH/*An*FNR/*An*Fd (*An*Fd, 5 μM) and (C), NADPH/*An*FNR/*An*Fld (*An*Fld, 3 μM). The corresponding insets show the evolution of the absorbance at 450 nm with the time. Absorbance spectra for the pre-

steady-state kinetically distinguishable species obtained by global analysis of the reactions with (D) boAdx, (E) *An*Fd and (F) *An*Fld. Insets of (D) and (E) show the evolution of these species along the time when the process is fitted to an A<>B>C model. Intermediate A, B and C species are denoted by bold, dotted and dashed lines, respectively. In (F) a more complex mechanism applies and the experimental results best fit to two superimposed processes, A<>B>C A plus A<>B'>C'. A, B and C' are shown as bold, dotted and dashed lines respectively.

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