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Valentina E. V. Ferrero, Giovanna Di Nardo, Gianluca Catucci, Sheila J. Sadeghi and Gianfranco Gilardi*

Cytochromes P450 are a superfamily of monoxygenases highly relevant for pharmaceutical, environmental and biocatalytical applications. The binding of a substrate to their catalytic site is usually detectable by UV-vis spectroscopy as a low-to-high spin state transition of the heme iron. However, the discovery of potential new substrates is limited by the fact that some compounds do not cause the typical spin shift even if they are oxidised by P450 enzymes.

Here we report a fluorescence-based method able to detect the binding of such substrates to the heme domain of cytochrome P450 BM3 from *Bacillus megaterium*. The protein was labeled with the fluorescent probe N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-Yl)-ethylenediamine (IANBD). Arachidonic and lauric acids are substrates of P450 BM3 and were used to validate the method, as their binding can be detected both by a spin shift of the Soret peak from 419 to 397 nm and by the fluorescence change of the labelled protein. The fluorescence emission of the probe linked to the protein increased by a value corresponding to 121 ± 9% and 52 ± 5% respect the initial one, upon titration with arachidonic or lauric acids respectively. The dissociation constants were calculated by both UV-vis and fluorescence spectroscopy. Three drugs, propranolol, chlorzoxazone and nifedipine, known to be oxidized by P450 BM3 and that bind without causing spin shift, were also tested and the fluorescence emission of IANBD was found to decrease by 29 ± 5%, 21 ± 2% and 23 ± 3% respectively, allowing the measurement of their dissociation constants.

Introduction

Cytochrome P450s are a wide superfamily of heme-thiolate enzymes of high biotechnological interest as they are able to degrade toxic xenobiotics, to metabolise drugs and to catalyse the production of high-value fine chemicals.[1] Over the last few decades many efforts have been directed to engineer their selectivity towards different compounds, such as drugs and environmental pollutants.[2-6] In particular, cytochrome P450 BM3 from *Bacillus megaterium,* a fatty acids monoxygenase,[7-9] is the P450 most widely used as biocatalyst for many reasons: it is a soluble fusion protein containing the heme and the reductase domains in a single polypeptide chain;[7] it is highly efficient both in substrate turnover and electron transfer;[10] it is often used as a model for mammalian P450s due to its sequence similarity to the human liver drug metabolizing enzymes.[11-13] Furthermore, it has already widely been shown to be an optimal target for protein engineering aimed at the generation of variants capable of industrially important transformations.[14-15] All these features highlight the importance to develop new experimental strategies to rapidly detect the binding of a new substrate to the active site of P450 BM3. Even if the binding of a substrate to cytochromes P450 is known to cause a low-to-high spin state transition of the heme iron, resulting in a shift in the position of the Soret peak,[16-18] in many cases such a spin shift is weak or not detectable[19-20] even though the substrate is turned over into its product. This behaviour is not well understood: the 3D structure of the heme domain of P450 BM3 (BMP) and selected mammalian P450 bound to their substrates showed that the distance between the ligand and the heme is too large for the mono-oxygenation reaction to occur, implying that some structural rearrangements must take place for the reaction to proceed.[21-23] Also the NMR spectra of reduced P450 BM3 suggested that the substrates are 6Å closer to the porphyrin ring than in the oxidized form, indicating that relevant changes occur upon heme reduction, allowing the substrate to move closer to the heme iron.[24-25] Although the substrate binding step is thought to take place before reduction of the iron, it has also been shown that substrates could bind and/or dissociate at different steps of the catalytic cycle.[26-27]

In the absence of a spin shift, the availability of a fast and easy method for measuring the binding of substrates with an estimate of their affinity for wild type or engineered cytochromes P450 is an attractive challenge in the bioanalytical field. For this reason, many groups have developed *in silico* methods able to predict substrate binding,[28-29] but a laboratory based, quick and easy assay is still necessary to experimentally screen possible ligands, avoiding lengthy incubations of the substrates with NADPH and cytochrome P450-reductase and product identification by HPLC-MS or GC-MS.[30-31]

Fluorescence spectroscopy is a sensitive technique widely used for ligand binding studies. A fluorescence-based method has been reported to detect the binding of new substrates to cytochrome P450 BM3.[32] This method is based on the inhibition of the reactions leading to fluorescent products.[32] On the other hand, cysteine-labeled proteins has been used for ligand binding studies and it has been successfully applied in many fields, ranging from the periplasmic binding proteins,[33-34] antibodies[35] and enzymes.[36] In particular, fluorescent probes have been attached to CYP2B4, CYP3A4 and P450eryF to study conformational rearrangements and cooperativity of binding sites in P450s.[37-39]

In this work a fluorescent probe was site-specifically introduced on BMP. The N,N'-Dimethyl-N-(Iodoacetyl)-N'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-Ethylenediamine (IANBD amide) is used to label the cysteines located in an allosteric site[36] by a thioether bond[35, 40-41] allowing detection of small conformational changes induced by substrate binding. This kind of studies have never

been performed on the P450 BM3 and an easy ligand binding assay could be very useful since this enzyme is considered as a model P450. The method is validated on both fatty acids and drugs.

In a previous work, P450 BM3 was found to turn over propranolol, chlorzoxazone and nifedipine.[14] These drugs are used in this work as BMP ligands because i) they do not cause a substrate induced spin shift and ii) they are very interesting for biotechnological applications such as the synthesis of human metabolites. Absorbance and fluorescence spectroscopy are used to detect the binding of fatty acids to the NBD-labeled BMP, and the resulting dissociation constants (K_D) are measured.

Experimental procedures

Materials

All chemicals were of reagent grade. All the solutions were prepared using milliQ water. All the resins used for protein purification were purchased by GE Healthcare. Most chemicals were purchased from Sigma-Aldrich: ampicillin, sodium dithionite, arachidonic acid, caffeine, chlorzoxazone, diclofenac, imidazole, lauric acid, nifedipine, pelargonic acid and propranolol. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Inalco and N,N'-Dimethyl-N-(Iodoacetyl)-N'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-Yl)-Ethylenediamine (IANBD amide) from Invitrogen. Fatty acids stock solutions were firstly prepared in ethanol and then diluted in 50 mM K_2CO_3 pH 8.0. Nifedipine was firstly prepared in methanol and then diluted with 100 mM potassium phosphate buffer (KPi) pH 7.4. The other compounds were directly prepared in 100 mM KPi pH 7.4, which is the buffer used for all the titration assays.

Mutants preparation, protein expression and purification

Single cysteine BMP mutants were engineered, expressed and purified as previously described.[42] Freshly transformed *Escherichia coli* BL21 (DE3) cells were grown in Luria-Bertani (LB) media with ampicillin (100 µg/mL) and induced with 1 mM IPTG. After induction, the cells were grown for 18 h at 28 °C and then disrupted by sonication. The protein was isolated and purified following previously published procedures.[43] The integrity of the heme thiolate bond of the purified protein was verified by carbon monoxide-binding assay. The protein was reduced by the addition of sodium dithionite and its UV-vis spectrum recorded with an Agilent 8453E spectrophotometer (Agilent Technologies). Then carbon monoxide was bubbled into the latter protein solution for a few minutes, and the resulting spectrum was recorded. The binding of carbon monoxide to the reduced form of the protein, causes the appearance of the characteristic 450 nm absorbance peak. Enzyme concentrations were calculated using the difference absorption method (A450 minus A490 for the reduced/CO bound minus reduced spectrum) and an extinction coefficient at 450 nm of 91000 $cm^{-1}M^{-1}$.[44]

Protein labeling

The purified protein (usually 100 µM) was incubated with a five fold excess of dithiothreitol for 30 min at 4 °C to reduce intermolecular disulphide bonds. The excess of dithiothreitol was then eliminated by a PD10 Sephadex G25 (GE Healthcare) gel filtration column. The fluorescent probe IANBD amide (absorption λ_{max} 478 nm, emission λ_{max} 540 nm) was then added in a ten fold excess (stock solution prepared in acetonitrile) and the mixture was incubated for 3 h at 4 °C. Then the sample was loaded on a PD10 Sephadex G25 column to separate the protein to the unreacted probe. The sample was eluted in 100 mM KPi buffer pH 7.4 and UV-vis spectra of the fractions were recorded to analyse the ratio between the concentration of the labeled protein (Cf) over the concentration of unlabeled protein (Cp). The Cf/Cp ratio indicates the labeling level and it was calculated as:

$$Cf / Cp = (A_{478} \cdot \varepsilon_{280\text{-BMP}}) / ((A_{280} \cdot \varepsilon_{478\text{-IANBD}}) - (A_{478} \cdot \varepsilon_{280\text{-IANBD}}))$$

where $\varepsilon_{280\text{-BMP}} = 117000\ M^{-1}\ cm^{-1}$, $\varepsilon_{478\text{-IANBD}} = 50300\ M^{-1}\ cm^{-1}$ and $\varepsilon_{280\text{-IANBD}} = 1745\ M^{-1}\ cm^{-1}$.

Circular dichroism measurements

Far UV and near UV-vis circular dichroism (CD) experiments were performed on the NBD-labeled and unlabeled BMP at room temperature. Four spectra were accumulated and averaged for each sample with a Jasco-J600 spectropolarimeter. The measurements were carried out using 5 µM of protein and quartz cuvettes with a pathlength of 0.1 cm for the far UV (200–250 nm) and 1 cm for the near UV (250–300 nm) and visible (300–600 nm) regions. The CD experiments were also performed in presence of 50 µM arachidonic acid and 5 mM propranolol.

P450 BMP substrate binding assay by UV-vis spectroscopy

Substrate binding was monitored using spectrophotometric titrations in a 1 cm path-length cuvette by following the characteristic low-to-high spin transition, as a shift in the main Soret absorption band from 419 to 397 nm.[17] The reaction mixture contained 1 µM concentration of enzyme in 100 mM KPi pH 7.4, and spectra were recorded after each substrate addition to the sample cuvette. All spectral titration were carried out at room temperature. The dissociation constant, K_D, was determined by fitting the absorbance differences against the concentration of free substrate, using the following equation:

$$(\Delta Abs_{397}\text{-}\Delta Abs_{419}) = ((\Delta Abs_{397}\text{-}\Delta Abs_{419})_{max} * [S]_{free}) / (K_D + [S]_{free})$$

where [S] is the substrate concentration, $[S]_{free}$ is [S]added-[ES]

$[ES] = (\Delta A_{394}\text{-}\Delta A_{419})[P450] / (\Delta A_{394}\text{-}\Delta A_{419})_{max}$.

Data were fitted using a one site saturation ligand binding curve using the SigmaPlot software.

P450 BMP-NBD substrate binding assay by fluorescence spectroscopy

The fluorescence emission spectra of the NBD-labeled proteins were recorded at room temperature with a fluorescence

spectrometer LS55 (Perkin Elmer). Emission spectra were obtained by excitation of the labelled proteins at 478 nm. The reaction mixture contains 1 μM concentration of enzyme in 100 mM potassium phosphate pH 7.4, and aliquots of ligands were added from the stock solution. Fluorescence spectra were recorded after each substrate addition to the sample cuvette (equilibration time of 1 min). Substrate binding was monitored observing the intensity change of the probe emission upon substrate additions. Binding curves were obtained by plotting the fluorescence intensity versus substrate concentration. K_D was determined by fitting the data using a one site saturation ligand binding curve using SigmaPlot software. Fluorescence data were fitted using the following equation:

$$\Delta F = \Delta F_{max} \cdot [S]_{free} / (K_D + [S]_{free})$$

where ΔF is the fluorescence intensity difference observed after each substrate addition and the reference spectrum with no substrate; ΔF_{max} is the maximum fluorescence intensity change derived from the fitting; $[S]_{free}$ is [S]added-[ES]; K_D is the dissociation constant of the substrate for the enzyme. Fluorescence data are expressed in percentage of change. Control experiments adding the only 50 mM K_2CO_3 pH 8.0 buffer were performed and a small progressive decrease of the fluorescence signal, corresponding to a maximum of 20% of the initial fluorescence intensity value, was observed. This decrease was used to correct all the fluorescence variation percentage values.

Protein – ligand in silico interaction analysis

The YASARA STRUCTURE package[45] was used to perform all the molecular docking and dynamics simulations. The ligand molecules were obtained from the Hic-UP[46] database and optimized using YAPAC (built-in geometry optimization program). Autodock[47] was employed to dock the optimized propranolol and arachidonic acid molecules to the 2IJ2 structure.[48] The binding sites for these molecules were selected based on the ligand binding pocket of cytochrome P450 BMP.[21] YASARA Global Docking macro performed 50 different runs of docking and saved the top docked structure applying a scoring value for the best binding energy. Two hundred and fifty runs of Local Docking were run and among the best poses the most suitable docking modes for propranolol and arachidonic acid were selected.

Molecular dynamics (MD) simulations and energy minimization of the ligand free, propranolol and arachidonic acid docked structures were then carried out using AMBER 03 (the same force field for all the previous analyses). MD simulations were carried out for 10 nanoseconds. Every 6250 simulation steps, with a timestep of 4 femtoseconds, a simulation snapshot was saved. The final output of the molecular dynamics resulted in a total of 400 trajectories that were analysed by computing the time average structure with B-factors calculated from the root mean square fluctuations. Each average structure was then utilized for the accessible molecular surface calculation by using WHAT IF server http://swift.cmbi.ru.nl/servers/html/index.html and the hydropathy score of the residues close to the nitrobenzoxadiazole moiety (calculated on http://gcat.davidson.edu/rakarnik/kytedoolittle).

Results and discussion

BMP labeling with IANBD AMIDE

The wild type BMP contains three cysteine residues, C62, C156 and C400, all located on the proximal side of the heme, away from the distal side that is involved in substrate binding (Figure 1A). While C400 is engaged as the heme 5th ligand, analysis of the X-ray 3D structure shows that C62 and C156 display a solvent exposed area of 11.6 and 1.0 $Å^2$ respectively, indicating that in principle only C62 is available for the covalent linkage with the fluorescent probe IANBD.

Mutants C62S and C156S, as well as the double mutant C62S/C156S, were used to test the ability to covalently link the thiol-specific and environment sensitive fluorophore IANBD amide. All proteins were heterologously expressed in *E. coli* and purified to reach a purity ratio (A_{419}/A_{280}) of 1.1-1.4, values that are consistent with pure holo-enzymes.[49-50] All samples showed a Soret peak at 419 nm that shifted to 450 nm upon reduction and binding of carbon monoxide, indicating the integrity of the heme thiolate bond.

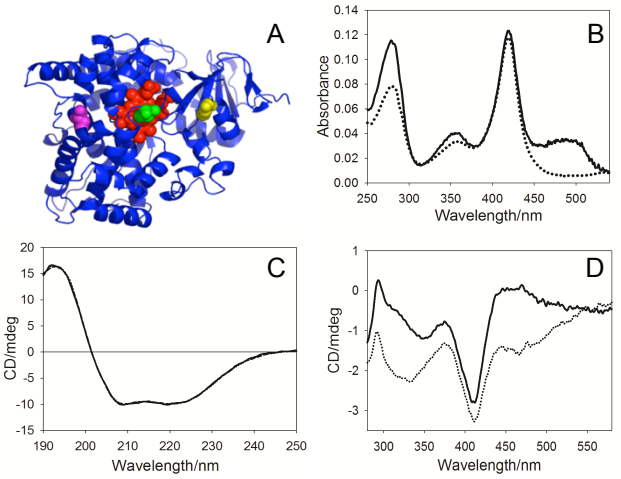

Fig. 1. A) Proximal side of the three-dimensional structure of wild type BMP (PDB file: 1 FAG). The heme is shown in red, the heme ligand C400 in green, C156 in magenta, and C62 in yellow. B) Typical absorbance spectra of 1 μM wild type (solid line) and C62S/C156S mutant (dotted line) labeled with IANBD. Differences in absorbance at 280, 350 and 478 nm are due to the presence of the protein-bound NBD. C) Overlay of the far-UV CD spectra of 5 μM wt before (solid line) and after (dotted line) labelling with IANBD. No significant differences are observed. D) Near-UV/vis CD spectra of 5 μM wt before (solid line) and after (dashed line) labeling with IANBD. Differences at 340 nm and 480 nm are due to the presence of the protein-bound NBD.

Prior to labelling with IANBD, the purified proteins were incubated for 30 minutes at 4 °C with a five fold excess of dithiothreitol. This reduces the intermolecular disulphide bridges responsible for protein dimerisation in the wild type and C156S mutant, as confirmed by native PAGE. Excess dithiothreitol was then removed by gel filtration prior to incubation with the proteins for 3 hours at 4 °C with a ten fold excess of IANBD, followed by a second gel filtration for the removal of excess fluorophore.

The presence of the covalently bound fluorescent probe was detected by visible spectroscopy at 478 nm (Figure 1B, solid

line). The labeling efficiency C_f/C_p were 0.88 ± 0.06 , 0.76 ± 0.08 and 0.23 ± 0.05 for wt, C156S and C62S respectively.

These values confirm that wt and C156S, carrying the most exposed C62, have a very similar labeling efficiency with one IANBD molecule bound per molecule of enzyme, while C62S and C62S/C156S, due to the low exposure to the solvent of C156 in C62S or the lack of exposed cysteines in C62S/C156S, show very low and no labeling, as confirmed by the lack of the 478 nm peak (Figure 1B, dotted line). These data indicate that the labeling occurs only on C62 even in the wt protein that therefore was used for the following analysis, while the mutant C156S/C62S was used as a negative control.

Far UV and Near UV-vis CD spectroscopy of labeled and unlabeled proteins

In order to verify that the binding of the IANBD label to the wt did not affect the overall fold of the protein, far-UV and near-UV CD spectra were recorded and compared to those of the unlabeled proteins (Figure 1C and D). As expected, the far-UV CD spectra showed a predominant α-helix secondary structure, as demonstrated by the presence of two peaks at 208 and 222 nm. Figure 1C shows the overlapping far-UV CD spectra of labeled (dotted line) and unlabeled (solid line) BMP demonstrating that the IANBD label did not interfere with the protein overall folding. The near-UV/vis spectra showed the presence of the IANBD probe on the protein due to the peaks at 340 and 480 nm (Figure 1D, dashed line) that are absent on the unlabelled protein (Figure 1D, black line).

Substrate binding studies by UV-visible and fluorescence spectroscopy

The binding ability of the un-labelled and NBD-labelled BMP for two fatty acids substrates (arachidonic acid and lauric acid) was followed by UV-vis absorption spectroscopy and the spectra obtained in the case of arachidonic acid are shown in Figure 2A and 2C.

The low-to-high spin transitions induced by addition of increasing amounts of arachidonic acid (from 0 to 30 μM) and lauric acid (from 0 to 400 μM) were monitored as a shift in the main Soret absorption band from 419 to 397 nm.

The apparent dissociation constants, K_D, were calculated by plotting the absorbance differences versus the substrate concentration and fitting the data to the hyperbolic function described in the material and methods section. The apparent K_D values for arachidonic acid and lauric acid are in the μM range in either cases (Table 1) and they are comparable to those reported in the literature.[51-52]

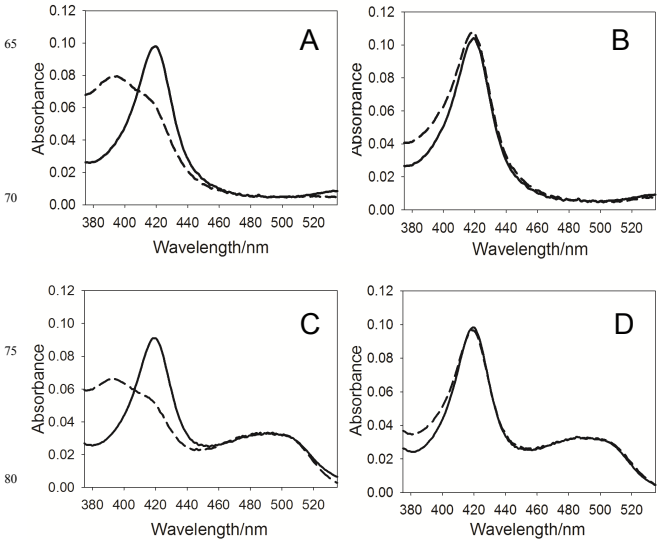

Fig. 2. Typical absorbance spectra of un-labeled BMP (A and B) before and after addition of arachidonic acid (A, solid and dashed line respectively) or propranolol (B, solid and dashed line respectively) and NBD-labeled wt (C and D) before and after addition of arachidonic acid (C, solid and dashed line respectively) or propranolol (D, solid and dashed line respectively).

These data confirm that the covalent linkage of the fluorescent probe to C62 does not affect the ability of the enzyme to bind the substrate. The same experiment performed by binding arachidonic acid to the C156S mutant led to apparent K_D values of 6.5 ± 0.4 μM and 3.9 ± 0.2 μM for the unlabelled and NBD-labelled enzymes respectively, in keeping with the values observed for the wt.

The labelled protein was then used for binding studies followed by fluorescence emission.

Table 1. Binding of substrates to BMP wt. Dissociation constants (K_D) are calculated from the UV-vis spin shift (NBD-labelled and un-labelled) and from the fluorescence emission changes (NBD-labeled) for the substrates arachidonic acid, lauric acid, propranolol, chlorzoxazone and nifedipine.

	Apparent K_D (μM)		
	UV-vis spin shift		Fluorescence emission
	Unlabeled	NBD-labeled	NBD-labeled
Arachidonic acid	5.6 ± 0.4	4.1 ± 0.7	0.7 ± 0.1
Lauric acid	100.0 ± 15.0	137.6 ± 28.0	129.7 ± 29.2
Propranolol	No spin shift	No spin shift	830.0 ± 120.0
Chlorzoxazone	No spin shift	No spin shift	890.0 ± 190.0
Nifedipine	No spin shift	No spin shift	106.5 ± 10.3

Figure 3A shows the increase in the fluorescence emission

intensity measured at increasing concentrations of arachidonic acid. Control experiments performed by additions of only the buffer (50 mM K_2CO_3 pH 8.0) to the labelled proteins showed an increase in fluorescence emission of 20% respect to the initial value. When arachidonic acid was added up to 30 μM, the fluorescence emission increased (Figure 3A). The fluorescence change, corrected for the buffer contribution (20%) corresponded to 121 ± 9% of to the initial value. Additions of arachidonic acid and lauric acid to free IANBD in the absence of the enzyme did not lead to changes in fluorescence emission.

Figure 3B shows the plot of the fluorescence emission intensity normalized as a percentage of the maximal value observed versus the substrate concentration. Fitting the data to a hyperbolic binding function confirmed a one-site ligand binding behaviour, as previously observed by UV-vis spectroscopy, with an apparent K_D of 0.7 ± 0.1 μM (Table 1). Experiments performed on the NBD-labeled C156S mutant led to an apparent K_D value of 1.6 ± 0.3 μM, in keeping with the wt and confirming that C62 is the labeled site.

Lauric acid, another fatty acid substrate of P450 BM3, was titrated in the range 0-400 μM leading to a net maximal 52 ± 5% fluorescence increase, confirming the behaviour observed for arachidonic acid. An apparent K_D of 129.7 ± 29.2 μM (Table 1) was calculated and this is in good agreement with literature reports of 100.0 ± 15.0 μM.[53]

The apparent K_D measured by fluorescence emission for arachidonic acid is one order of magnitude lower than that measured by UV-vis spectroscopy. The two methods follow different events caused by substrate binding: UV-vis spectroscopy looks at the change in the heme iron spin caused by release of the water 6th ligand triggered by substrate binding, while fluorescence spectroscopy is sensitive to the changes in the protein environment around the probe, triggered by indirect structural rearrangements in the protein scaffold caused by substrate binding. The reason for the apparent higher affinity of the NBD-labelled enzyme can be explained with additional interactions deriving from the probe, as already reported for other labeled proteins.[33, 54]

In a previous study, we demonstrated that wild type P450 BM3 is able to turn over propranolol, chlorzoxazone and nifedipine performing different kind of reactions, including hydroxylation and dealkylation.[14] Unlike arachidonic acid,[55] these drugs were found not to induce a spin shift when added to both the labeled and unlabeled proteins, as shown in Figure 2B and D for propranolol, and for this reason they were selected for binding experiments to be followed by fluorescence emission. Spectra were collected using an excitation at 478 nm and following emission at 540 nm, specific for the IANBD probe, with the scope of exploiting the sensitivity of this probe towards changes in the microenvironment that occur upon substrate binding.

Fluorescence emission was also studied during the binding of propranolol, a non-selective beta-blocker drug used in the treatment of hypertension.[5] Figure 3C shows that when propranolol (from 0 to 1.5 mM) was added to the labeled protein, the fluorescence emission intensity decreased by 49 ± 5% (Table 2). Figure 3D shows the binding curve calculated from the titration of BMP with propranolol. Also in this case the data points were fitted to a hyperbolic function from which an apparent K_D value of 830 ± 120 μM was calculated (Table 1).

The apparent K_D obtained for propranolol is in the high μM range, in keeping with previous kinetic experiments based on the measurement of product formation,[14] while the apparent K_D values measured for arachidonic acid are in the low μM, that is consistent with the fact that arachidonic acid is a good substrate of P450 BM3. A control performed using free IANBD in the absence of the enzyme did not show changes upon addition of increasing amounts of propranolol.

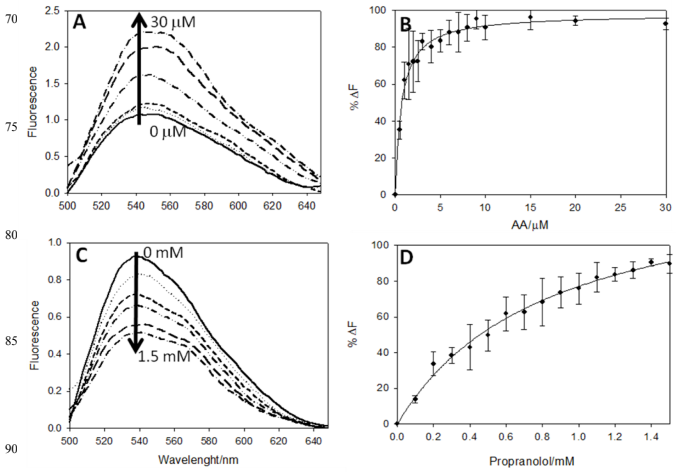

Fig. 3. A) Fluorescence emission spectra of 1 μM wt BMP labeled with IANBD upon addition of increasing concentrations of arachidonic acid. B) Binding curve of arachidonic acid to 1 μM NBD-labeled wt; data are normalized to the maximum value of fluorescence emission. C) Emission spectra 1 μM wt labeled with IANBD upon addition of increasing concentrations of propranolol. D) Binding curve of propranolol to 1 μM NBD-labeled wt.

The same binding experiments were performed with the other drugs and an inhibitor, and the results are summarized in Table 1 and Table 2. Chlorzoxazone, a muscle relaxant used to treat spasm and pain,[56-57] known to give an hydroxylated reaction product,[14] showed a net decrease of 21 ± 2% in the fluorescence emission when titrated in a 0-3 mM range of concentrations, confirming the behaviour of propranolol. In this case a 41% decrease of fluorescence was observed at the maximum substrate concentration of 3 mM, then corrected for the buffer contribution (Table 2) and the apparent K_D was 890 ± 190 μM (Table 1).

Nifedipine, a dihydropyridine calcium channel blocker used as an anti-anginal and anti-hypertensive[58] was also titrated into BMP-NBD in a range of 0-500 μM leading to a decrease in fluorescence by 23 ± 3%, (Table 2), once corrected for the buffer contribution, with an apparent dissociation constant of 106.5 ± 10.3 μM (Table 1). When imidazole (IMI), a known inhibitor of P450 BM3,[59] was tested, despite a shift in the absorption spectra from 419 to 424 nm, no significant net change was observed in fluorescence emission. The decrease in fluorescence emission observed titrating this molecule from 0 to 5 mM was 20%, comparable to what observed when titrating the buffer (Table 2). This result is consistent with the observation from the available crystallographic data for N-(12-imidazolylldode-canoyl)-L-leucine-bound BMP show a structure very similar to the substrate free form.[60] Diclofenac and caffeine were used as negative controls as they are known not to be substrates of P450 BM3.[14] Titration with these molecules, both in a 0-2 mM range, gave rise

to a decrease of fluorescence intensity in the range of the buffer control (20%) and were corrected to zero (Table 2).

Table 2. Maximal fluorescence emission variations observed for substrates capable or incapable of inducing spin-shift, non substrates and inhibitor. Control experiments were carried out by addition of only 50 mM K_2CO_3 buffer pH 8. The fluorescence emission variations are expressed as a percentage with respect to the initial intensity observed prior to addition of the molecule tested.

Sample	Molecule	UV-vis spin shift (nm)	Fluorescence emission variation (%)
Buffer control	K_2CO_3 50mM	None	0
Inhibitor	Imidazole	419 → 424	0
Non-substrate	Diclofenac	None	0
	Caffeine	None	0
	Pelargonic acid	None	0
Substrates	Arachidonic Acid	419 → 397	121 ± 9
	Lauric Acid	419 → 397	52 ± 5
	Propranolol	None	- 29 ± 5
	Chlorzoxazone	None	- 21 ± 2
	Nifedipine	None	- 23 ± 3

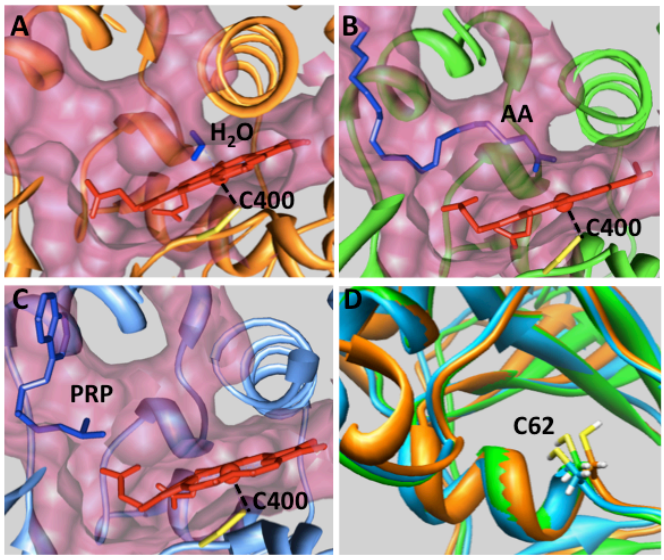

Fig. 4. A) Surface area of the active site (magenta) of substrate-free wt (PDB file 2IJ2, orange ribbon). The heme is represented in red sticks, the C400 heme-ligand in yellow, and sixth-heme ligand water molecule bound to the heme-iron is shown in blue. B) Active site of wt docked with arachidonic acid (blue). C) Active site surface area of wt docked with propranolol (blue). D) Superposition of the 3D models around the C62 residue (yellow sticks) in the substrate free (orange ribbon), arachidonic acid- (green ribbon) and propranolol- (cyan ribbon) bound forms. Note the different orientations of the thiol group of C62.

Since arachidonic and lauric acids have a detergent-like structure that might affect the protein conformation, a further control was performed with pelargonic acid, a fatty acid that is not oxidised by P450 BM3 and does not induce a spin shift.[7-9] Titration of this molecule (0-1.3 mM range) into BMP-NBD gave rise to the fluorescence linear decrease of 20% as the other controls.

Analysis of the spectra of Figure 2 shows that in all cases substrate binding does not alter the absorbance at the excitation wavelength of IANBD (478 nm). This result rules out that the absorbance changes due to the spin shift are responsible for the different behaviour observed between fatty acids and drugs. On the other hand it is known that IANBD is an environment-sensitive probe. The 3D structure of BMP has shown that binding of substrates induces conformational changes.[24-25] The fluorescence data suggests that these conformational changes can be divided into two groups that generate differences in the environment experienced by the probe, reflecting the different conformation rearrangements of the protein while binding different molecules with diverse properties.

Another route through which even small conformational changes can affect the quantum yield of the fluorescence probes such as IANBD is the twisted intra-molecular charge transfer.[61] Here the charge-transfer groups such as the donor tertiary amino-group and the acceptor nitro-group belonging to IANBD, relax efficiently when co-planar. As previously observed for other ligand-binding proteins,[54] small conformational changes in the protein can affect the IANBD intra-molecular charge transfer leading to changes in the fluorescence emission intensity.

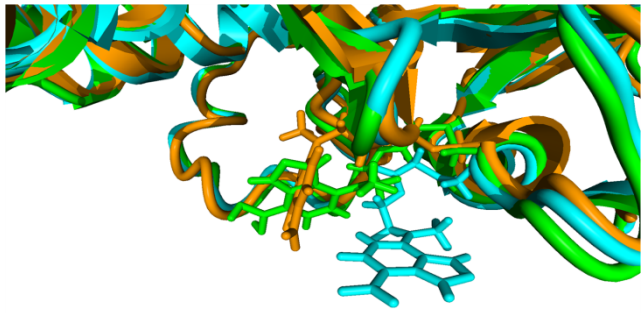

Fig. 5. Superposition of the 3D models of the wt protein substrate-free (orange ribbon) and docked with arachidonic acid (green ribbon) and propranolol (cyan ribbon). The NBD moiety bound to the cysteine 62 is represented in sticks (orange for substrate-free, green for arachidonic acid-bond and cyan for propranolol-bound). Note the different positions of the NBD moiety in the three situations.

The possibility that small conformational changes are at the basis of different fluorescence responses was investigated by *in silico* docking calculations with the YASARA software using the BMP structure in the substrate-free form (Figure 4A, PDB: 2IJ2). Molecular dynamics and energy minimization of the ligand-free, arachidonic acid- and propranolol- docked structures (Figure 4A, 4B, 4C) were then carried out using the AMBER 03 software. The binding energy of 7.71 and 6.06 kcal/mol calculated with the software YASARA for arachidonic acid and propranolol respectively, nicely reflect the trend in affinity experimentally measured by the K_D values. Figure 4D shows the movements experienced by C62 in the substrate-free structure (orange), in the docked models of arachidonic acid-protein (green) and propranolol-BMP complexes (cyan). Modeling simulations clearly show movements in the C62 thiol to which the NBD fluorophore is attached.

The analysis of the molecular dynamics trajectories shows that when the NBD probe was attached to C62, different conformations in the NBD position are found as shown in Figure 5. In particular, when propranolol is bound, NBD is highly

exposed to the solvent (35 % exposure), while when arachidonic acid is bound, the exposure is decreased (24 %). The analysis of the nature of the amino acid side chains found within a sphere of 4 Å radius around the NBD probe, shows a different pattern of residues deriving from the conformational changes induced by substrate binding. The hydrophobicity scores of these residues found for the arachidonic acid bound enzyme are -0.77, while for the propranolol complex these values are -1.81. Furthermore, a conformational change that brings the fluorophore in closer contact with water molecules (as in the propranolol case) will decrease the fluorescence signal, while a conformational change that shields it from the solvent (as with arachidonic acid) will increase the fluorescence.

The possibility that fluorescence changes observed are associated to quenching/de-quenching due to Föster resonance energy transfer (FRET) or electron transfer to chromophores or aromatic residues[62-64] has also been taken into account. The overlap between the emission spectrum of IANBD and the absorption spectrum of BMP, both in absence and presence of arachidonic acid, is shown in Figure S1. Differences between substrates that induce or not a spin shift are present in the spectral region ranging from 510 to 600 nm (α and β bands). These differences might also contribute to the fluorescence changes observed.

Conclusions

In conclusion, this work shows how an environment-sensitive fluorescence probe such as IANBD, covalently linked to cytochrome P450 BMP, can function as a reporter group for binding of both physiological and non-physiological substrates. Significant changes in fluorescence emission, varying from 19 to 130% of the initial values not only allows to screen for binding, but also to determine the apparent dissociation constant K_D. It can be expected that this approach could be applied to other P450 enzymes as well as other proteins where the binding of a ligand causes a small conformational rearrangement. The approach allows to easily screen for binding without addition of reagents but simply titrating the molecule under investigation, prior to lengthy and costly turnover studies with NADPH followed by LC-MS experiments. Only the molecules proving positive to binding can then be further investigated by LC-MS with an important reduction of work and costs. Nevertheless, the possibility to have false negative from substrates that do not induce a fluorescence change when binding to BMP, has to be taken into account.

This fluorescence-based strategy is a potential tool for ligand screening studies aimed at screening for new substrates for P450 enzymes for biotechnological applications.

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Notes

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‡ Abbreviations:
BMP: heme domain of cytochrome P450 BM3
C62S: BMP cysteine 62 mutated to serine
C156S: BMP cysteine 156 mutated to serine
C62S/C156S: BMP double mutant
IANBD amide: N,N'-Dimethyl-N-(Iodoacetyl)-N'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-Yl)-Ethylenediamine
NBD: nitrobenzoxadiazole moiety
nss: no spin shift

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