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Cytochrome P450 BM3, NO Binding and Real-Time NO Detection**

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Abbreviations:
P450-BM3$_{oxy}$, the isolated oxygenase domain of cytochrome P450 BM3; nNOS, rat neuronal nitric oxide synthase; P450 Cam, *campylobacter* P450; *E. coli, Escherichia coli*; DTT, Dithiothreitol; H4B, (6R)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; EDTA, ethylenediaminetetra-acetate; EGTA, etheleneglycoltetra-acetate; MOPS, 3-N-morpholinopropanesulfonic acid; Tris, tris (hydroxymethyl)aminomethane.

Keywords:
nitric oxide; Cytochrome P450; monooxygenation; binding constant; detection; assay; UV/Vis spectroscopy

Abstract

Nitric Oxide is known to coordinate to ferrous heme proteins very tightly, following which it is susceptible to reaction with molecular oxygen or free NO. Its coordination to ferric heme is generally weaker but the resultant complexes are more stable in the presence of oxygen. Here we report determination of the binding constants of Cytochrome P450 BM3 for nitric oxide in the ferric state in the presence and absence of substrate. Compared to other 5-coordinate heme proteins, the K_d values are particularly low at 16 and 40 nM in the presence and absence of substrate respectively. This most likely reflects the high hydrophobicity of the active site of this enzyme. The binding of NO is tight enough to enable P450 BM3 oxygenase domain to be used to determine NO concentrations and in real-time NO detection assays, which would be particularly useful under conditions of low oxygen concentration, where current methods break down.

Introduction

Cytochrome P450 BM3 is an unusual self-sufficient fatty acid monooxygenase from *Bacillus megaterium* [1; 2]. It comprises an N-terminal domain containing a typical Cys-ligated heme, which is fused to a C-terminal reductase domain. The latter is closely related by sequence, structure and function to mammalian cytochrome P450 reductase and binds FAD, FMN and NADPH [3]. This arrangement enables the enzyme to catalyse NADPH dehydrogenation at the FAD site, electron transfer via an anionic FMN semiquinone [4] and fatty acid hydroxylation at the heme site, all in a single component system, rather like the mammalian NO synthases[5]. A few other fused P450 systems have been discovered [6; 7], but they are rare and P450 BM3 is certainly the most widely known. Recently these have been exploited in biotechnological applications such as electrode immobilised biosensors [8], whole cell biotransformations and screening [9]. X-ray crystal structures have been determined for the different component domains of P450 BM3 [10], and for the isolated heme domain (P450-$BM3_{oxy}$) with a substrate bound [11]. As a result P450-$BM3_{oxy}$ is one of the most highly characterized of all P450s. One of the advantages of using P450 BM3 as a model P450 system is its ease of use, it exhibits high solubility and stability, atypical of this family of enzymes, and assays are greatly simplified by the fused-reductase. The isolated heme domain (P450-$BM3_{oxy}$) is particularly stable and remains functional when supplied with a suitable source of electrons [12]. The substrate specificity of the enzyme is determined by the active site pocket, which is long and narrow [11]. Near the substrate entry point is the Arg 47 residue, which recognises the carboxylate group of the substrates (Fig. 1) [13]. A hydrophobic channel then extends to the heme distal face to accommodate a long aliphatic chain. Near the heme iron, Thr268 is situated to participate in the proton transfer steps necessary for activation of dioxygen [14], a component conserved in virtually all P450s. Apart from the few hydrophilic residues involved in this process the active site is hydrophobic, and has been shown

to catalyse efficient alkane hydroxylation [15] with minor modification. Despite this, purified P450-$BM3_{oxy}$ coordinates a water molecule as a 2nd axial ligand, and may accommodate a further one within the active site cavity [16]. Substrate binding displaces the water and induces a low to high spin-state change [11]. The more effective the substrate, the more complete the spin state shift observed [17]. Thus far, arachidonic acid (C20) appears to be the most effective substrate, although palmitoleic acid (C16) bound at the active site is the best so far characterized structurally (Fig 1) [11]. The substrate-induced spin-state shift is known to trigger heme reduction by facilitating electron transfer from the FMN [18]. It also increases the reduction potential of the heme [17]. In the absence of substrate, the heme reduction potential is particularly low. This is attributable in part to the heme thiolate ligand, which is both a strong σ and π donor. Other factors are likely to include the general hydrophobicity of the heme binding site. Mutation of residues on the proximal side of the heme has been shown to induce wide swings in the reduction potential by changing the polarity of the environment [18; 19]. The axial ligand effect is likely to induce a higher electron density on the heme iron, facilitating oxygen activation during catalysis and the binding of π acceptor ligands such as O_2, CO and NO, particularly in the ferrous oxidation state. Among these three similar ligands, NO is the only one able to bind to ferric heme proteins. However, its binding affinity is usually several orders of magnitude lower for the ferric state than the ferrous, although heme *d* appears to have evolved to favour NO dissociation from the ferrous state [39, 40]. Table 1 shows the range of binding affinities for a selection of well-characterized heme complexes and proteins [20; 21]. The Cys-thiolate ligated heme proteins appear to have the highest NO affinity, NO synthase and campylobacter P450 (P450 Cam). However, in routine titrations, we noticed that the binding of NO to ferric P450-$BM3_{oxy}$ was particularly tight. In this paper we determine the dissociation constant for the ferric NO complex and describe how P450-$BM3_{oxy}$ can be used to assay the concentration of NO in a solution, either to calibrate a stock solution or in a real-time assay for NO production.

	K_d (μM)	**Reference**
$[Fe^{III}(H_2O)(TPPS)]^{3-}$	900	[21]
MetMyoglobin	71	[21]
MetHemoglobin	250	[36]
Catalase	5.5	[21]
NO synthase	2.5	[25]
P450cam	0.56	[37]
P450 BM3oxy	0.016 0.042	This study

Table 1. Dissociation constants for Nitric Oxide and a Selection of Ferric Heme Proteins.

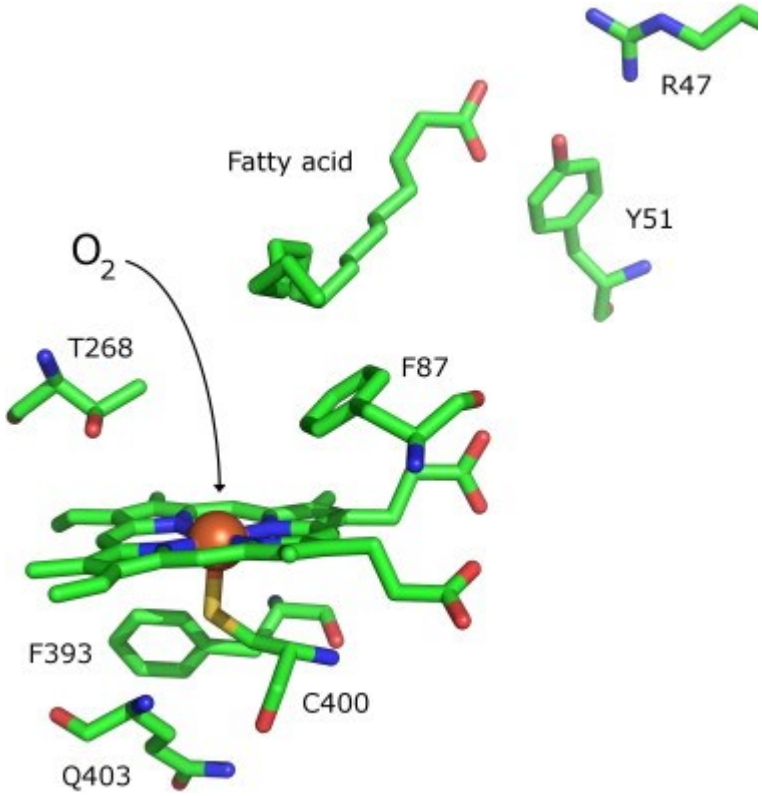

Figure 1. The active site of P450 BM3 with a fatty acid substrate (palmitoleic acid) bound [11]. Produced from pdb file 1FAG using PyMol [38].

Experimental Procedures

Materials: Unless otherwise stated, all materials were purchased from Sigma UK

***Enzyme Preparations*:** P450-BM3$_{oxy}$ was over-expressed in *E. coli* cells using a pCW$_{ori}$ expression vector and purified as described previously [22], concentrated to >500 μM by ultrafiltration and flash frozen in liquid nitrogen prior to storage at -80 °C. Rat neuronal NO synthase (nNOS) was over-expressed in *E. coli* cells using a pCW$_{ori}$ expression vector and purified using ADP-sepharose and CaM-agarose affinity columns as described previously [23]. Following gel filtration to remove EGTA and concentration by ultrafiltration to 100 μM, it was stored at -80 °C in the presence of 10 μM H_4B (Schricks Lab, Jona, Switzerland) and 1mM DTT. Recombinant bovine calmodulin was also purified from *E. coli* cells as described previously [24]. The resulting solution in 50 mM Tris/HCl, 1 mM $CaCl_2$ was lyophilized and stored at -20 °C.

Spectrophotometric Analysis of Imidazole and Nitric Oxide Binding to P450-BM3$_{oxy}$: UV/visible absorption spectra were recorded over the 300-800 nm range using a Cary 50 spectrophotometer and quartz cuvettes of 1 cm path length containing 1-5 μM P450-BM3$_{oxy}$ in 1 ml of assay buffer (100 mM

MOPS pH 7.0) at 30 °C. Imidazole dissociation constants were determined by titration of small aliquots of a concentrated solution of imidazole into the cuvette. The change in the Soret absorbance peak was measured from the maximum to minimum of the difference spectrum after each addition. These values were plotted against concentration and fitted to a rectangular hyperbolic function by non-linear regression analysis using Origin 8.0 (Microcal).

Titrations with nitric oxide were performed using small aliquots taken from a saturated solution of NO in the assay buffer. This was prepared by gently bubbling 10 ml of anaerobic buffer (taken from a glovebox) for 5 minutes with NO gas (98.5 % Aldrich) in a septum-sealed tube, with a small bubbler connected to the gas exit needle to prevent oxygen ingress [25]. The NO titrations were performed entirely within a Belle Technology anaerobic glove box with an oxygen concentration below 10 ppm. In the presence of imidazole the data were treated as described above to give apparent dissociation constants for NO with P450-$BM3_{oxy}$ at each imidazole concentration. In the absence of imidazole binding was too tight.

***NO Synthesis Assay*:** A sample of P450-$BM3_{oxy}$ was passed through an anaerobic G25 gel filtration column (1.5 x 10cm) and eluted into 50 mM Tris/HCl buffer pH 7.5 containing 100 mM NaCl. A 1 cm, 1 ml quartz cuvette was filled with a 10 μM sample of the protein diluted in the same buffer. The assay mixture also contained 100 μM NADPH, 1 mM L-arginine, 10 μM H_4B, 1 mM DTT, 1 mM $CaCl_2$ and 10 μg/ml calmodulin, all added from concentrated stocks. 5 μl of air-saturated buffer was added, followed by 50 nM nNOS. The absorbance change at 437 nm was monitored using a Cary 50 UV/Visible spectrophotometer. The entire procedure was carried out in a Belle Technology anaerobic glove box with oxygen concentration below 10 ppm.

Results

Determining the concentration of NO in a saturated solution: In order to generate the ferric-NO complex of P450-$BM3_{oxy}$, enzyme was made anaerobic by passing through a G25 gel-filtration column within an anaerobic glove box. A 1 ml sample of approximately 5 μM in 0.1 M MOPS buffer pH 7 was titrated with a saturated solution of nitric oxide (1 μl additions) in the same buffer causing a shift in the spectrum from 418 to 438nm (Figure 2) or from 390 nm to 438 nm in the presence of substrate (arachidonate) as shown by Quaroni *et al.* [26]. The ferric NO complex remained stable and the spectrum was unaltered after 1 hour under these conditions. In both cases the resultant spectral changes were almost stoichiometric in nature, exhibiting curvature only when the saturation point was reached. Consequently it was clear that NO binding was unusually tight for a ferric heme complex, the K_d values for which commonly lie in the μM range (Table 1).

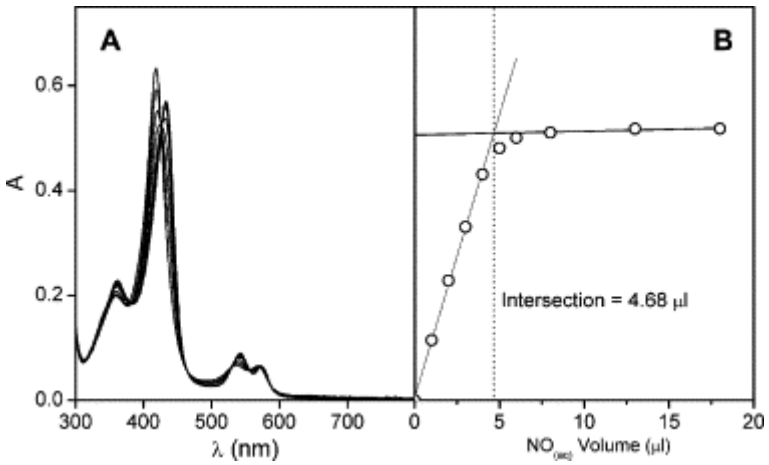

Figure 2. Titration of ferric P450 BM3oxy with 1µl aliquots of saturated NO solution. Panel A, changes to the UV/Visible spectrum. Panel B, plot of volume added versus maximum spectral change observed: ΔA(438 nm) - ΔA(418 nm).

In fact this forms a convenient method for measuring the concentration of NO in a saturated solution (Figure 2). For P450-$BM3_{oxy}$ the concentration of a 1 ml solution was determined using the extinction coefficient of 108 $mM^{-1}cm^{-1}$ at 419 nm. Titration with µl aliquots of a saturated NO solution and plotting yielded the saturation curve shown in Figure 2B. Both the increasing and horizontal linear phases were then fitted to straight line functions. The resulting intercept in Figure 2B shows that in this case 4.7 µl of NO saturated solution were required to fully bind the P450-$BM3_{oxy}$ in 1 ml of a 6.43 µM solution, giving an NO concentration of 1.37 mM. We have now adopted this method as a routine assay [25].

Determination of the dissociation constants for imidazole: Due to the strength of NO binding to P450-$BM3_{oxy}$ it was necessary to use a competitive inhibitor in order to measure the K_d value for the ferric NO complex. In this case imidazole was used. Imidazole is known to coordinate to the ferric heme iron of P450 BM3, shifting the Soret absorbance peak from 390 or 419 nm in the presence or absence of substrate respectively to 425 nm, inducing a shift to low spin. In order to use imidazole as a competitive inhibitor of NO binding to the heme it was necessary to determine the binding constants for imidazole in both cases. Figure 3 shows the titrations of P450-$BM3_{oxy}$ with imidazole in Panels A and B. The resultant absorbance changes were plotted against imidazole concentration and fitted to a simple rectangular hyperbolic function to give the K_d values. In the absence of substrate K_d = 236 µM, in the presence of 100 µM arachidonate K_d = 390 µM.

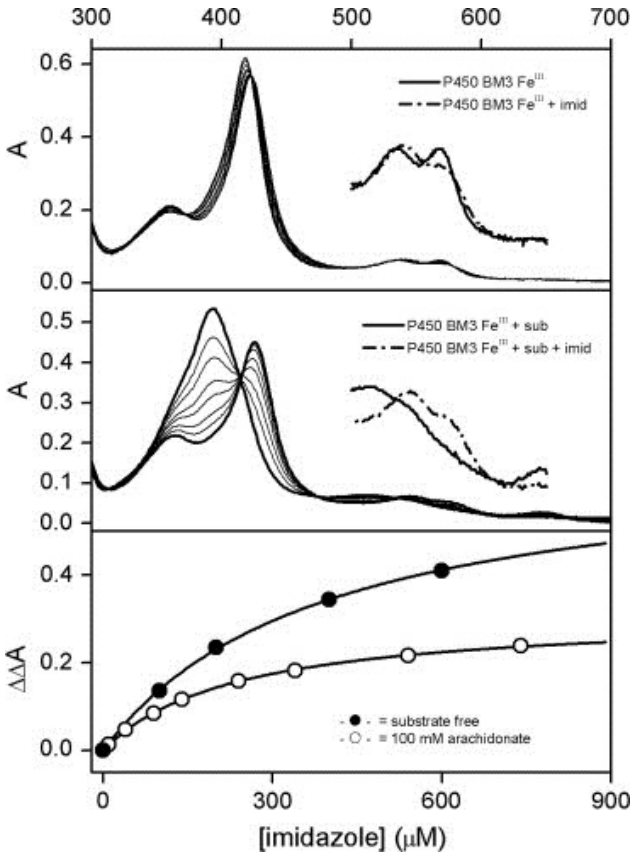

Figure 3. UV/Visible spectral titration of ferric P450-BM3$_{oxy}$ with imidazole in the absence (A) and presence (B) of substrate (arachidonate). Plots of the change in absorbance measured from the maximum and minimum in the difference spectra are shown in (C) fitted to rectangular hyperbolic fitting functions to determine dissociation constants.

Determination of dissociation constants for NO: Using different concentrations of imidazole to inhibit the binding of NO, samples of P450-BM3$_{oxy}$ were titrated with aliquots from a NO solution of known concentration. Sets of titrations were performed in the absence and presence of 100 μM arachidonate. The data for these are shown in Figures 4 and 5. In both cases NO bound to the ferric heme displacing imidazole and shifting the Soret band from 425 nm to 438 nm. Higher concentrations of imidazole increased the concentration of NO required to achieve saturation. At each imidazole concentration a binding curve was plotted and K_d value calculated for the P450-BM3$_{oxy}$ ferric NO complex (Table 2). The K_d values were plotted against imidazole concentration (not shown) for both datasets, yielding straight lines intercepting very close to the origin. It is clear that the inhibitory effect of imidazole approximates to being competitive, but that the K_d values are too small to enable accurate determination using the graphical method. However, by assuming competitive binding it is possible to directly determine the corrected K_d values at each imidazole concentration by using K_d(observed)/K_d(NO) = [imidazole]/K_d(imidazole) + 1. The average of these three values is as

follows: in the absence of substrate K_d = 42 (± 17) nM, in the presence of substrate K_d = 16 (± 1) nM (included in Table 1).

[imidazole] (mM)	$K_{d\ (NO)}$ (μM)	
	+ substrate	- substrate
2.1 mM	0.1	0.67
21 mM	0.82	2.50
42 mM	1.73	5.67

Table 2. Dissociation constants for Nitric Oxide and P450-BM3oxy Determined in the Presence of Different Concentrations of Imidazole.

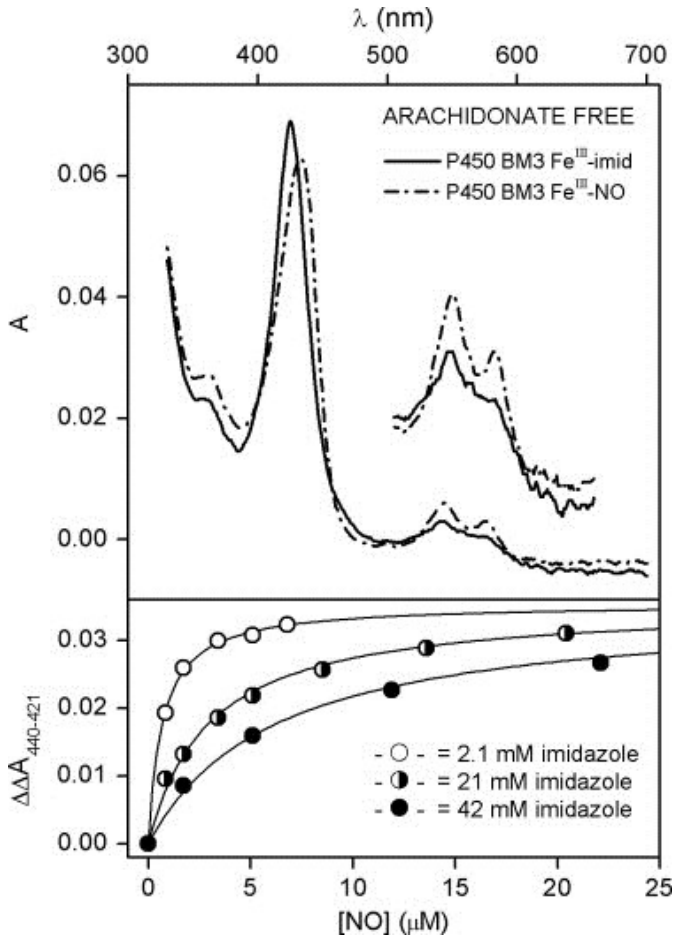

Figure 4. UV/Visible spectral titration of ferric P450-$BM3_{oxy}$ with NO in the presence of imidazole. Panel A shows the resultant spectral changes. Panel B shows the difference in absorbance at 421 and 440 nm plotted for imidzole concentrations of 2.1, 21 and 42 mM on titration with NO. Data were fitted to rectangular hyperbolic fitting functions to determine dissociation constants.

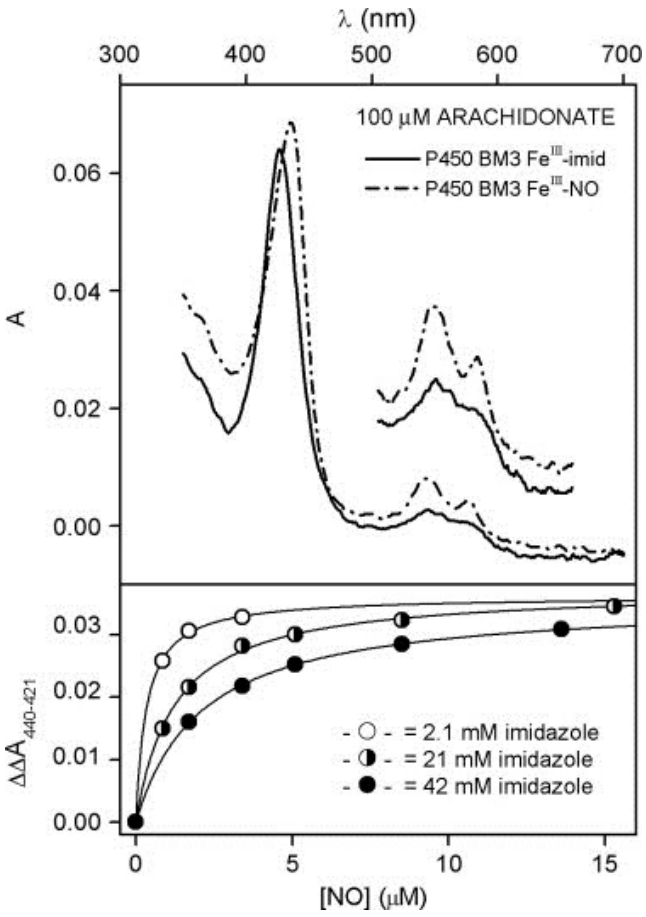

Figure 5. UV/Visible spectral titration of ferric P450-BM3$_{oxy}$ with NO in the presence of imidazole and 100 μM Arachidonate. Panel A shows the resultant spectral changes. Panel B shows the difference in absorbance at 421 and 440 nm plotted for imidzole concentrations of 2.1, 21 and 42 mM on titration with NO. Data were fitted to rectangular hyperbolic fitting functions to determine dissociation constants.

Using P450 BM3 to detect NO in a real-time assay: In order to test whether P450-BM3$_{oxy}$ can be used to detect nitric oxide produced spontaneously, a real-time assay was performed. A 1 ml sample of approximately 10 μM was prepared in an anaerobic box and placed within a spectrophotometer in a 1 cm quartz cell. The assay solution included 100 μM NADPH, 1 mM L-arginine, 10 μM H_4B, 1 mM DTT, 1 mM $CaCl_2$ and 10 μg/ml calmodulin. The assay mix was stable and no change in the spectrum of the P450 occurred during preparation or subsequent incubation. To initiate turnover, 1 μM O_2 from an air-saturated solution was added to the assay mix along with 50 nM neuronal NO synthase. This resulted in an immediate increase in absorbance at 436 nm, indicating formation of the ferric NO complex of P450-BM3$_{oxy}$. The increase continued in a linear time-dependent way for 2 to 3 minutes (Figure 6). The assay therefore functions as expected, with NO quantities in the low nM region being detected. This resolution range is very similar to that of the widely used oxyhaemoglobin assay [27],

which is functional only at oxygen concentrations high enough to saturate the hemoglobin. NO reacts irreversibly with oxyhemoglobin to generate nitrate, whereas in the P450-$BM3_{oxy}$ assay the NO is simply bound tightly to the heme. For the latter, a low concentration of NO will persist in solution, and under some circumstances this could be viewed as a disadvantage. However, with 10 μM P450-$BM3_{oxy}$ and 0.1 μM NO produced in the assay, this would be substantially less than 1 nM, given the K_d values determined above. It is widely known that NO acts as a feedback inhibitor of NO synthase [28], caused by a K_d value of 0.17 nM [25] for the ferrous NO complex. Thus significant build-up of NO would have caused rapid inhibition of NO synthesis in the above assay, resulting in curvature of the assay trace.

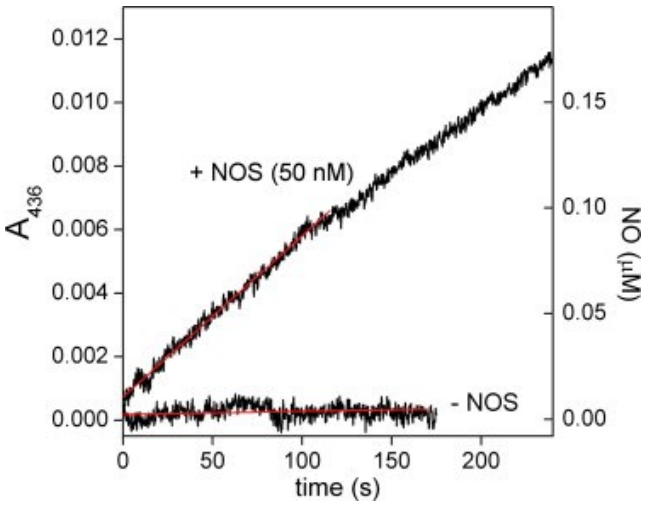

Figure 6. Assay of NO synthesis activity by nNOS under microaerobic conditions. The mixture contained 10 μM P450-$BM3_{oxy}$, 1 mM L-Arginine, 0.1 mM NADPH , 1 mM $CaCl_2$,10 μg/ml Calmodulin, 1 μM oxygen

Discussion

For ferric heme complexes and proteins the dissociation constants vary from around 1 mM to 0.5 μM (Table 1). In many cases the NO ligand must compete with water for the heme iron coordination site. Indeed in many crystal structures water molecules can be observed in this position. Nitric oxide tends to be bound in a more linear orientation with Fe(III) as compared to with Fe(II) and is considered to be a 3-electron donor. Consequently the Ferric NO complex is unlikely to be stabilised by H-bonding to the distal oxygen atom, whereas this is the case for ferrous NO complexes [29]. Another factor to consider is the axial heme ligand. Nitric oxide can be considered to be an electron deficient free-radical, it therefore has higher affinity for electron-rich metal centres, favouring Fe(II) over Fe(III). It

is also likely therefore to favour the electron rich centres of P450s, which are characterized by cysteinyl thiolate axial ligands. Consequently, it is no surprise that ferric P450 Cam also has a high NO binding affinity. P450 BM3 combines the axial ligand effect with a hydrophobic active site, which has evolved to bind the long aliphatic chains of fatty acids. It is interesting that the substrate-bound form actually binds NO more tightly, presumably due to the increase in local hydrophobicity. Thus it appears that the combination of a thiolate axial ligand and hydrophobic active site leads to unusually high NO affinity for ferric heme and suggests a strategy for designing heme-based NO sensors. We show that P450-$BM3_{oxy}$ can be used as a convenient method for calibrating the concentration of a saturated solution of NO prior to use. Secondly we show that under anaerobic or microaerobic conditions P450-$BM3_{oxy}$ detects nM concentrations of NO produced in a real time assay. Currently, commonly used assays for NO detection are chosen to suit the particular experiment. These include: (1) the oxyhemoglobin assay [30], used for monitoring NO production by enzymes (2); NO sensor electrodes [31], which can monitor NO concentrations down to nM concentrations; (3) the Griess assay, which actually determines nitrite and nitrate concentrations [30]; (4) detection using fluorescent reagents such as 4,5-diaminofluorescein [32], which are commonly used on whole cell and tissue samples, but are less useful for quantitative work; and (5) the chemiluminescent reaction of gaseous NO with ozone [30], useful for measuring NO in the headspace above a reaction solution. In order to accurately monitor the rate of NO production by an enzyme or system, the most robust quantitative method is the oxyhemoglobin assay. Oxyhemoglobin reacts rapidly and irreversibly with NO [33] to form nitrate and the change in visible absorbance is monitored. NO does not therefore accumulate in solution (as in methods 2, 3 and 5) and cause unwanted side effects such as enzyme inhibition or modification. The main drawbacks are that the assay will only work under aerobic conditions, and that the ferrous form of hemoglobin is required, which is known to catalyse NO generation from nitrite [34]. This is a likely problem with all ferrous heme proteins, which also bind NO tightly, but tend to have lower NO-complex stabilities. There is substantial interest in the biosynthesis of NO under anaerobic or microaerobic conditions, from various sources including nitro-compounds and nitrite [35]. The ferric form of P450-$BM3_{oxy}$ is already a useable NO sensor for calibrating a stock solution of NO. In order to be effective in a real-time NO production assay, it must bind the NO essentially irreversibly. As shown above, for quantities up to 0.1 μM, the accumulated NO in solution would be less than 1 nM at equilibrium. It may also be possible to enhance the NO binding affinity of P450-$BM3_{oxy}$ by site directed mutagenesis. It would be relatively easy to design the active site to be even more hydrophobic by removing the catalytically important residues, which are not required in this application. Expression in *E. coli* and purification of the enzyme are relatively straightforward and high yield, although the protein is not currently being produced commercially.

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