Subzero-temperature stabilization and spectroscopic characterization of homogeneous oxyferrous complexes of the cytochrome P450 BM3 (CYP102) oxygenase domain and holoenzyme

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Abstract:

We describe herein for the first time the formation and spectroscopic characterization of homogeneous oxyferrous complexes of the cytochrome P450 BM3 (CYP102) holoenzyme and heme domain (BMP) at —55 °C using a 70/30 (v/v) glycerol/buffer cryosolvent. The choice of buffer is a crucial factor with Tris [tris(hydroxymethyl)aminomethane] buffer being significantly more effective than phosphate. The oxyferrous complexes have been characterized with magnetic circular dichroism spectroscopy and the resulting spectra compared to those of the more well-characterized oxyferrous cytochrome P450-CAM. The formation of a stable substrate-bound oxyferrous CYP BM3 holoenzyme, despite the fact that it has the necessary reducing equivalents for turnover, indicates that electron transfer from the flavin domain to the oxyferrous center is very slow at this temperature. The ability to prepare stable homogeneous oxyferrous derivatives of both BMP and the CYP BM3 holoenzyme will enable these species to be used as starting materials for mechanistic investigation of dioxygen activation.

Keywords:

Mono-oxygenase; Dioxygen activation; Oxyferrous heme iron; Magnetic circular dichroism; Cryoenzymology; Cysteinate-ligated heme iron; Cytochrome P450 BM3

Article:

The cytochrome P450 (P450) superfamily, consisting of cysteinate-ligated heme iron mono-oxygenases, is extensively distributed throughout the plant, animal, and microorganism kingdoms. The reactivity of this family covers a vast number of dioxygen activation reactions including hydroxylation of unactivated alkanes, epoxidation of olefins, and the conversion of aromatics to phenols and of sulfides to sulfoxides or sulfones [1–3]. The crucial oxygen atom insertion process in P450 is initiated by the binding of dioxygen to the ferrous iron [4,5] to yield the oxyferrous enzyme (1), in resonance with the ferric superoxide (2) complex (Fig. 1). Single electron reduction then gives the peroxoferric state (3), followed by proton addition to generate the hydroperoxoferric species (4). Addition of a second proton releases a water molecule to yield the oxo-iron(IV)-porphyrin radical cation state known as P450 compound I (5).

Oxyferrous P450 is quite unstable and readily autooxidizes to form the ferric state and superoxide anion. The rate of this process in the best characterized (and most stable) oxyferrous P450 derivative, P450-CAM, is about 0.01 s^{-1} ($t_{1/2}$ = ~69 s) at 25 °C [6]. The released superoxide

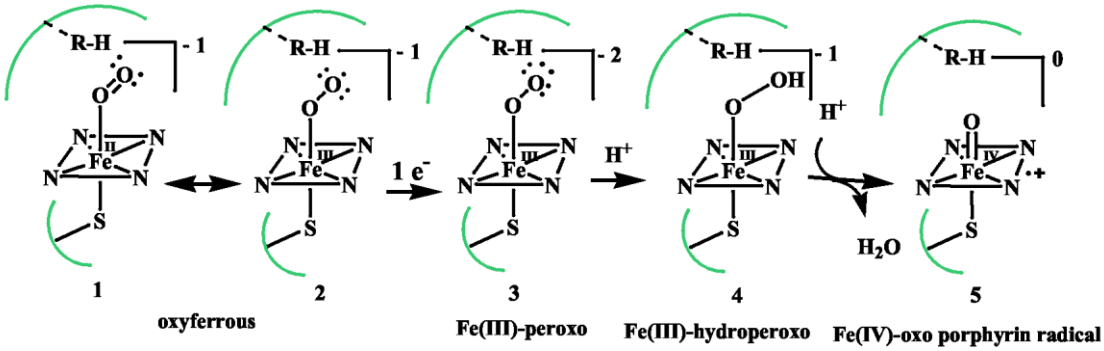

Fig. 1. Transient oxygen-containing intermediates in the cytochrome P450 reaction cycle.

disproportionates to generate hydrogen peroxide, a source of destructive hydroxyl radicals.

The fatty acid hydroxylase from Bacillus megaterium, P450 BM3, is a self-sufficient class II P450 composed of a heme-binding domain (BMP) and a flavin domain that contains both FMN and FAD. The X-ray structure of the ferric heme iron in substrate-free BMP (Fig. 2) reveals an iron-cysteinate Fe–S bond of 2.25 Å [7,8]. The formation of substrate-free and -bound oxyferrous BMP has been previously reported [9,10]. However, the results described herein demonstrate that the previously reported oxyferrous derivatives were significantly contaminated with ferric protein formed by autooxidation. In the present study, we describe for the first time the formation of stable homogeneous oxyferrous complexes of both BMP and the P450 BM3 holoenzyme. The homogeneity of these derivatives is demonstrated by comparison of their magnetic circular dichroism (MCD) and electronic absorption (EA) spectra to those of the more thoroughly characterized oxyferrous adduct of P450-CAM [1 1]. The ability to prepare stable homogeneous oxyferrous BMP and P450 BM3 holoenzyme will enable these species to be used as starting materials for mechanistic studies of dioxygen activation.

MATERIALS AND METHODS

Materials.

O_2 gas was obtained from Matheson. Sodium dithionite, arachidonic acid, glycerol, and all other reagent grade chemicals were purchased from Aldrich or Sigma and used without further purification. Sodium dithionite was kept in a desiccator under N_2 to maintain its effectiveness as a reductant.

P450 BM3 heme domain and holoenzyme.

The preparation and purification of P450 BM3, heme domain, and holoenzyme, from Escherichia coli clones containing plasmids for the respective proteins, has been described earlier [12,13]. The purified protein was stored at -70 °C in 300 mM Tris [tris(hydroxymethyl)aminomethane] buffer (pH 8.0). Concentrations of ferric BMP and the holoenzyme were determined using $\varepsilon_{417.5} = 100$ (ferric substrate-free) and $\varepsilon_{418.5} = 110$ mM^{-1} cm^{-1} (ferric substrate-free), respectively [14,15], at 4 °C in a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0). Buffer pH was measured at 4 °C in the absence of glycerol.

Spectroscopic techniques.

Electronic absorption (EA) spectra were recorded with a Varian-Cary 400 spectrophotometer (at ~4 °C) or a

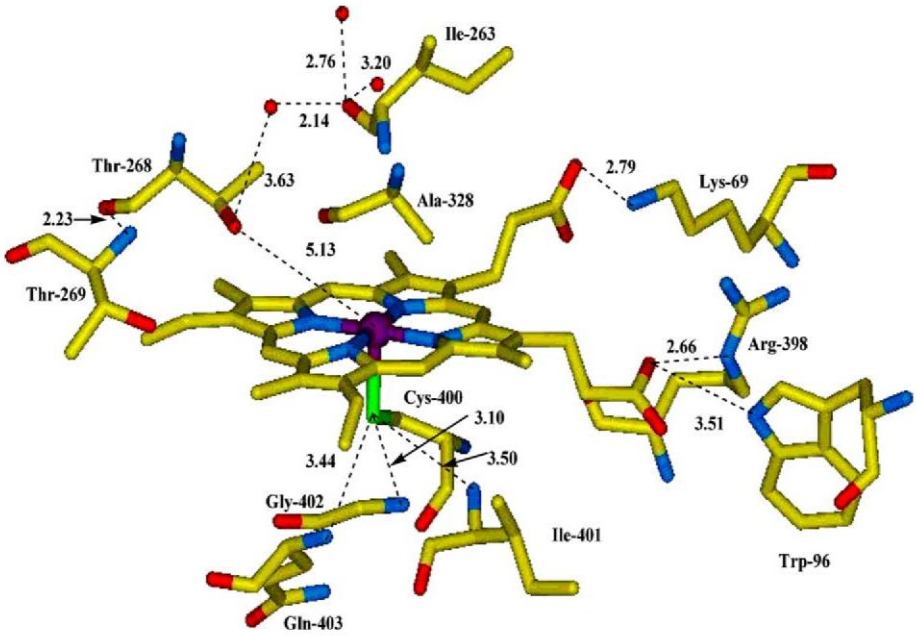

Fig. 2. The active site structure of ferric cytochrome P450 BM3 (CYP102) heme domain (PDB code 1BVY) [8]. The dashed lines and values indicate selected interatomic distances in Å.

JASCO J600A spectropolarimeter (at 55°C). Magnetic circular dichroism (MCD) spectra were measured in a 0.2 or 0.5 cm cuvette at a magnetic field of 1.41 T with the JASCO J600A spectropolarimeter as previously described [16]. The Jasco J600A spectropolarimeter is equipped for the simultaneous recording of both MCD or circular dichroism (CD) and EA (as transmission) spectra. Data manipulations (transmission to absorption conversion, baseline and natural CD subtraction, smoothing, and normalization) were conducted (see [16] for MCD data) with JASCO software.

Preparation of oxyferrous complex.

Oxyferrous complexes were generated at —50 to —60°C in a chest freezer in a mixed cryosolvent consisting of a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0). The ferric enzyme was first reduced by microliter additions of a near minimal amount of $Na_2S_2O_4$ (up to 0.5 mM from a 20 mg/mL stock) under N_2 at 4 °C in a sealed cuvette to generate deoxyferrous P450, followed by cooling to —50°C and then by thorough bubbling with pre-cooled O_2. Upon further cooling to temperatures as low as —55 to —60 °C, the glycerol-containing cryosolvent behaves like a semi-solid glass.

RESULTS AND DISCUSSION

Spectroscopic characterization of cytochrome P450 BM3 in the presence of the cryosolvent

The electronic absorption (EA) spectral properties of cytochrome P450 BM3 were first examined in the presence of the cryosolvent consisting of a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0). Addition of a slight excess of arachidonic acid substrate (55 µM) to the ferric P450 BM3 heme domain (BMP) (~50 µM) caused the partial displacement of the bound water molecule in the active site, generating a high-spin/low-spin mixture. As seen in Fig. 3, the Soret EA peak shifted from ~417.5 nm to a double-hump envelope maximized at ~396 nm, due to the high-spin component, but still retained significant absorption under the original peak from the low-spin component. A new peak appeared at 647 nm that could be assigned to the high-spin component [17]. The existence of a high-spin/low-spin equilibrium that depends on the substrate concentration is believed to be accompanied by rearrangements of pivotal water molecules and the hydrogen bond network in the active site region [18]. Similarly, with microsomal P450 2B4 in the presence of benzphetamine, a “ternary” complex of ferric heme iron, substrate, and a water molecule bound directly to the heme iron is known to exist [19].

Interestingly, further addition of excess substrate (~6 mM) gave mostly low-spin complex (Fig. 3) with the Soret EA peak at ~416 nm, with the usual peaks in the visible region at ~532 and ~565 nm attributable to a low-spin ferric heme center. The MCD spectrum of this species is shown in Fig. 4A. The spectrum is very similar to that previously reported for low-spin ferric P450-CAM [20] and consists of a moderately intense derivative-shaped feature in the Soret region and a series of increasing intense MCD peaks at 450, 510, and 550 nm, followed by a somewhat more intense, but negative, feature (trough) at 573 nm.

The EA and MCD spectra of the cytochrome P450 BM3 holoenzyme were generally similar to those of the corresponding BMP states, as illustrated with low-spin substrate-free ferric P450 BM3 holoenzyme (Figs. 4A and B). The presence of the flavin domain had a small effect on the EA spectrum (additional shoulder at ~475 nm) and almost no effect on the MCD spectrum of the low-spin ferric holoenzyme compared to the parallel derivatives of ferric BMP. Similar results have been reported for neuronal nitric oxide synthase [21].

Spectroscopic characterization of the oxyferrous complex of the oxygenase domain of cytochrome P450 BM3 (BMP)

A variety of conditions were examined in order to optimize formation of oxyferrous P450 BM3. The best cryosolvent was found to be a 70/30 (v/v) mixture of glycerol and

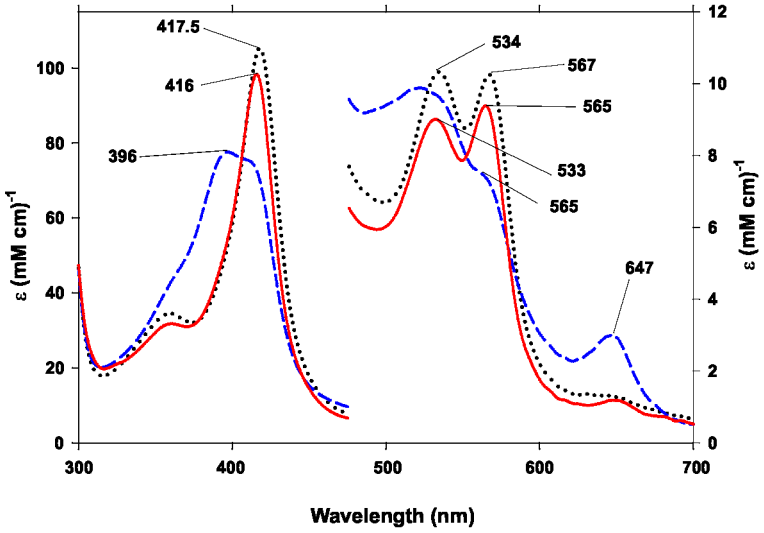

Fig. 3. Electronic absorption spectra at 4 °C of ferric BMP with and without substrate (arachidonic acid) bound: in the presence of 6.6 mM arachidonic acid-bound (solid line) and 57 μM arachidonic acid and (dashed line); ferric in the absence of substrate (dotted line). Samples were examined in a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0).

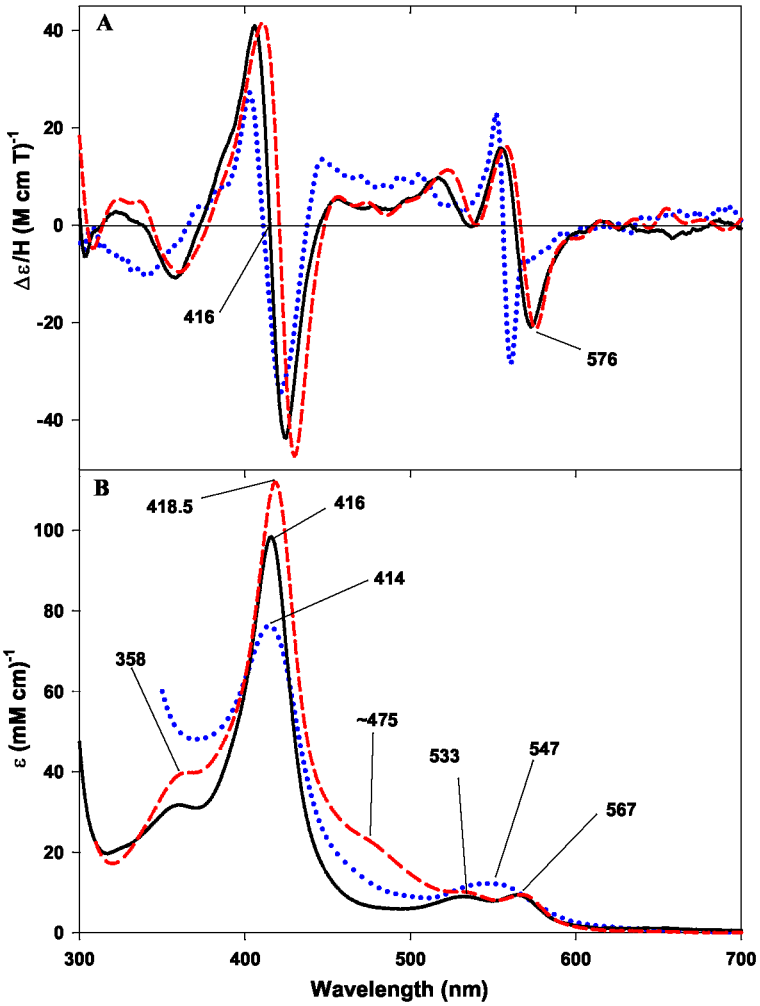

Fig. 4. Magnetic circular dichroism (A) and electronic absorption (B) spectra of ferric (solid line) and deoxyferrous (dotted line) 6.6 mM arachidonic acid-bound BMP and of substrate-free ferric BM3 holoenzyme (dashed line). Samples were examined in a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0) at −55 °C (A) and 4 °C (B). The EA spectra at −55 °C (not shown) were similar to those at 4 °C except for appearance of small peaks at 534 and 554 nm for the deoxyferrous form at −55 °C.

300 mM buffer (pH 8.0). The use of glycerol as the antifreeze solvent worked especially well since at temperatures as low as —55 to —60 °C, it behaved like a semi-solid glass. Because it has generally been found that substrate-bound oxyferrous P450 is more stable than the substrate-free complex [11], we examined various P450 BM3 substrates (arachidonic, lauric, and palmitic acids) and found that arachidonic acid had the most stabilizing effect. All attempts to prepare homogeneous oxyferrous BMP in the 70/30 cryosolvent mixture with 300 mM potassium phosphate (pH 7.7) were unsuccessful (data not shown) due to rapid autoxidation of the unstable oxyferrous complex, even at such low temperatures as —55 to —60 °C. Fortunately, Tris buffer was found to have a strong stabilizing effect on the oxyferrous state of P450 BM3.

Upon anaerobic reduction of the ferric arachidonic acid- bound BMP with sodium dithionite, the Soret EA peak at 417.5 nm shifted to 414 nm and a broad single peak appeared at 547 nm in a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0) (Fig. 4B). The MCD spectrum of ferrous substrate-bound BMP at —55 °C had a sharp derivative-shaped component centered at 556 nm (Fig. 4A) that was likely due to a small amount of six-coordinate ferrous species, as we reported for ferrous substrate-free P450-CAM under similar conditions [11]. The addition of pre-cooled oxygen generated a stable oxyferrous BMP that had EA and MCD spectra that were similar to the corresponding spectra of oxyferrous P450-CAM (Fig. 5) [11]. Oxyferrous BMP had a Soret EA peak at 422 nm and a single peak in the visible region at 559 nm; it had a prominent MCD trough at 587 nm.

The EA spectrum of oxyferrous BMP in the absence and presence of the substrate has been reported using stopped flow-rapid scan spectroscopy in 100 mM Mops [3-(N-morpholino)propanesulfonic acid] buffer (pH 7.0) at 15 °C by Ost et al. [22]. Because of rapid autooxidation of the oxyferrous complex (Soret EA peaks at 423 nm for the substrate-free and 425 nm for the arachidonic acid-bound forms), under these conditions, it is clear that only a mixture of oxyferrous (≤75%) and ferric form was observed

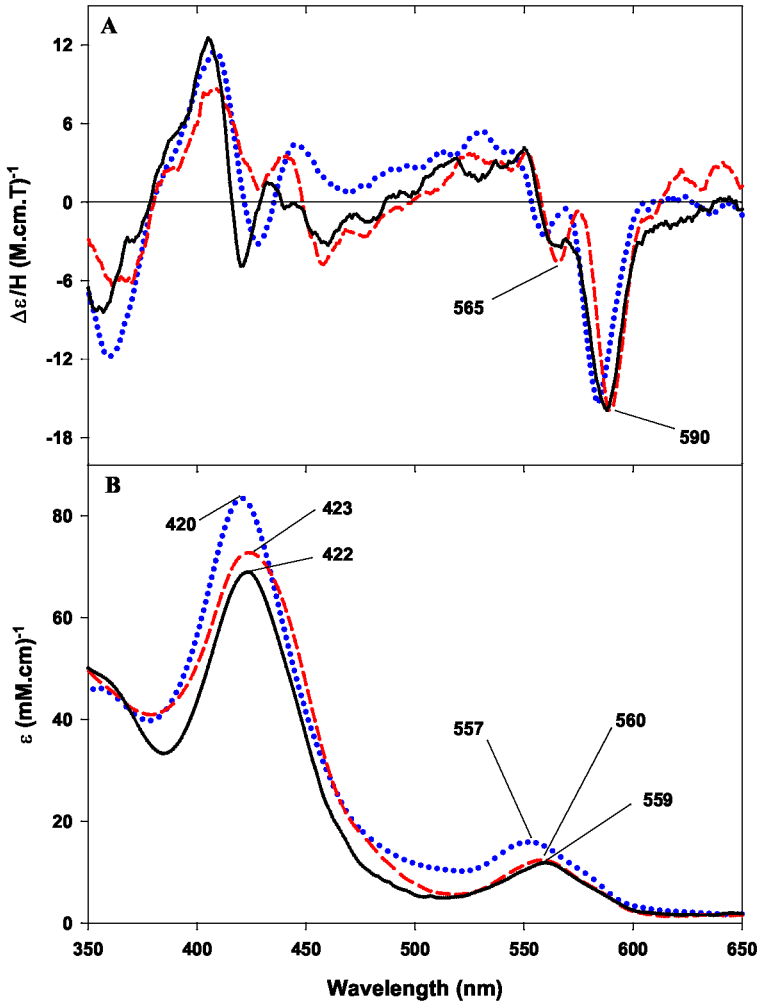

Fig. 5. Magnetic circular dichroism (A) and electronic absorption (B) spectra of oxyferrous complexes at −55 °C of BMP with 6 mM arachidonic acid-bound (solid line), of cytochrome P450 BM3 holoenzyme with 3 mM arachidonic acid-bound (dashed line), and of oxyferrous P450-CAM (dotted line). Samples were examined in a 70/30 (v/v) mixture of glycerol and 300 mM Tris buffer (pH 8.0). The spectra of oxyferrous P450-CAM are replotted from data in [11].

for the substrate-free state. At cryogenic temperatures (-25 °C, with 50% v/v ethylene glycol), less than 50% of the oxyferrous intermediate was seen by Bec et al. (estimated from Fig. 1 in [10]).

Hemoglobin and myoglobin form very stable oxyferrous complexes and function as molecular oxygen carriers. It has long been known that the autoxidation of oxyhemoglobin is promoted by anions, including phosphate [23,24]. Consequently, the observation reported herein that the two oxyferrous cytochrome P450 BM3 derivatives were much less stable in phosphate buffer is not surprising. In addition, the proximal ligand in globins is neutral histidine, a relatively poor electron donor compared to the thiolate proximal ligand found in all cytochrome P450 enzymes. The electronic “push” [25] of the proximal thiolate ligand will convey more charge onto the dioxygen ligand [i.e., an increased ferric-superoxo (2) contribution]. It is reasonable to suggest that the cationic nitrogen component of the Tris buffer may provide some additional charge neutralizing effect to stabilize the oxygenated complexes of the heme environment. We have recently found this to be a general phenomenon for thiolate-ligated oxyferrous heme centers [26].

Spectroscopic characterization of oxyferrous complex of the holoenzyme of cytochrome P450 BM3

Despite the fact that the P450 BM3 holoenzyme is composed of the heme-binding domain (BMP) and a flavin domain having both FMN and FAD bound, and therefore contains all of the necessary reducing equivalents for substrate turnover when totally reduced, we were able to generate a stable oxyferrous derivative at cryogenic temperatures. Fig. 5 shows the MCD and EA spectra of the arachidonic acid-bound BM3 holoenzyme at —55 °C in a 70/30 mixture of glycerol and 300 mM Tris buffer (pH 8.0). The MCD and EA spectra of the oxyferrous holoenzyme are similar to those of oxyferrous BMP (see below). Further, the presence of the sharp MCD trough at —590 nm indicates that the both oxyferrous P450 BM3 species did not contain any significant amount of the ferric (autoxidized) enzyme, which has an equally sharp MCD trough at ~576 nm (Fig. 4A) that is clearly missing. In addition, the EA spectra of both oxyferrous BM3 derivatives have a single peak in the visible region [559 nm (BMP) and 560 nm (holoenzyme)] (Fig. 5) that is distinct from the pair of peaks at 533 and 567 nm seen in the visible region EA spectrum of the low-spin ferric enzyme (Fig. 4). The MCD spectrum of the substrate-bound oxyferrous BM3 holoenzyme is also similar to that of the camphor-bound oxy-P450-CAM, with the main difference being the presence of a broad trough at ~455 nm in the spectrum of the holoenzyme. The formation of a stable substrate-bound oxyferrous CYP BM3 holoenzyme, despite the fact that it has the necessary reducing equivalents for turnover, indicates that electron transfer from the flavin domain to the oxyferrous center is very slow at this temperature.

Spectral comparison of the oxyferrous states of BMP and the P450 BM3 holoenzyme

Fig. 5A shows the comparison of the MCD spectra of oxyferrous BMP with that of the oxyferrous holoenzyme of cytochrome P450 BM3. The minor spectral difference at 455 nm between the spectra of the oxyferrous complexes of arachidonic acid-bound BMP and holoenzyme could be due to the influence of the flavin domain on the active site of the holoenzyme. Both oxyferrous BMP and the oxyferrous holoenzyme have asymmetric derivative-shaped Soret features. Additional overall spectral similarities with minor differences can be seen in EA spectra (Fig. 5B) with Soret peaks at ~423 nm and visible maxima at ~560 nm. Therefore, it can be concluded that the flavin domain has only a minimal effect on the spectral properties of the oxyferrous intermediate.

CONCLUSIONS

Characterization of the chemical and physical properties of the oxygenated intermediate in the P450 catalytic cycle is very important in order to achieve a better understanding of the nature of the reaction mechanisms of the P450 mono-oxygenases. We have found that preparation of stable oxyferrous P450 BM3 complexes not only depends on the temperature and handling method, but, especially, on the choice of the buffer. Herein, we have successfully generated and characterized the homogeneous oxyferrous complex for both cytochrome P450 BM3 oxygenase domain (BMP) as well as the oxyferrous state of the BM3 holoenzyme. Furthermore, we find that the flavin domain has only a minor influence on the spectral properties of the oxyferrous state.

The ability to prepare stable homogeneous oxyferrous forms of both BMP and the CYP BM3 holoenzyme will enable these species to be used as starting materials for mechanistic investigation of dioxygen activation. In particular, the successful stabilization of an extremely unstable (in potassium phosphate buffer) oxyferrous state will make it possible to carry out `cryoreduction' [4,5] experiments to capture and characterize oxygenated intermediates in the P450 catalytic cycle (Fig. 1, 3 and 4). In addition, with the oxyferrous holoenzyme, which has all of the necessary reducing equivalents for turnover, it may be possible to directly follow turnover by EA spectroscopy by slowly raising the temperature to “turn on” electron transfer.

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