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Short communication

Cobaltocene-mediated catalytic monooxygenation using holo and heme domain cytochrome P450 BM3

Andrew K. Udit *, Frances H. Arnold, Harry B. Gray *,[1]

Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 E California Blvd, Pasadena, CA 91125, USA

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Abstract

The feasibility of replacing NADPH with 1,1′-dicarboxycobaltocene in the catalytic cycle of cytochrome P450 BM3 has been explored. Using the holoprotein, the surrogate mediator was observed to reduce both the FAD and FMN in the reductase domain, as well as the iron in the heme domain. In an electrochemical system, the mediator was able to support lauric acid hydroxylation at a rate of 16.5 nmol product/nmol enzyme/minute. Similar electron transfer and catalysis were observed for the heme domain alone in the presence of the metallocene; the turnover rate in this case was 1.8 nmol product/nmol enzyme/minute. Parallel studies under the same conditions using a previously reported cobalt sepulchrate mediator showed that the two systems give similar results for both the holoenzyme and the heme domain.

Keywords: Cytochrome P450 BM3; Mediator; Electrochemistry; Biocatalysis

Cytochromes P450 perform a wide array of selective oxygenations under physiological conditions [1–3]. We are investigating soluble flavocytochrome P450 BM3 (BM3), a 119 kDa fatty acid hydroxylase from *Bacillus megaterium* containing a heme domain and an FAD/FMN reductase domain on a single polypeptide chain [4]. BM3 catalyzes hydroxylation at rates up to 1000 times higher than other P450s [5,6], while mutants of BM3 are even more active and have broader substrate specificity [7]. The enzyme has been well studied, resulting in a partial crystal structure [8] and extensive biochemical characterization [9].

Standing in the way of commercial use of BM3 and other P450s for selective oxidations is the requirement for reducing equivalents from NAD(P)H. NAD(P)H is expensive, decomposes over time, and is difficult to recover once oxidized. Attempts to regenerate NAD(P)H include chemical, electrochemical, enzymatic, and in vivo recycling systems [10–13]. Among the investigations seeking alternative reductants for P450 [14–16], the most successful utilized a platinum electrode and cobalt(III) sepulchrate (Co(sep)) as the electron shuttle [17] (Fig. 1(a)). Co(sep)-mediated catalysis was demonstrated with a variety of P450s, with rates approaching that of NAD(P)H-driven systems (110 vs. 900 min^{-1} for BM3 with lauric acid and either Co(sep) or NADPH, respectively) [17]. Practical limitations of the Co(sep) system, however, include production of reactive oxygen species [18], difficulty in synthetically manipulating Co(sep) to tune the mediator

* Corresponding authors. Tel.: +1-626-395-4205; fax: +1-626-568-8743 (A.K. Udit), Tel.: +1-626-395-6500; fax: +1-626-449-4159 (H.B. Gray).
E-mail addresses: andrewu@its.caltech.edu (A.K. Udit), hbgray@caltech.edu (H.B. Gray).
[1] Present address: Beckman Institute 139-74, California Institute of Technology, Pasadena, CA 91125, USA.

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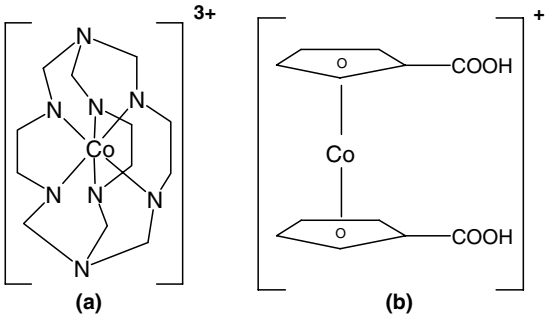

Fig. 1. Structures of: (a) cobalt(III) sepulchrate, and (b) 1,1′-dicarboxycobaltocene cation.

to different reaction conditions (e.g., solvent and pH), and aggregation and precipitation at functional Co(sep) concentrations (typically, 1–10 mM) [19].

We have been searching for better mediators for BM3. Previous work with glucose oxidase utilized a ferrocene derivative to mediate enzyme oxidation [20], demonstrating the idea of designing a mediator for a given application. For BM3 reduction, we decided to use the analogous reductant cobaltocene as a scaffold to construct a suitable mediator. In the hope that the dicarboxy derivative of cobaltocene could improve water solubility and disfavor aggregation, as well as raise the redox potential so the mediator would not be as air-sensitive, we synthesized 1,1′-dicarboxycobaltocenium hexafluorophosphate (M_{ox}) (Fig. 1(b)). [2] At physiological pH (7–8), the carboxyls are fully deprotonated [21], greatly enhancing water solubility. Cyclic voltammetry with a glassy carbon electrode in 0.1 M phosphate buffer pH 8 revealed that the Co^{III}/Co^{II} couple is fully reversible with an $E_{1/2}$ of −830 mV (vs. Ag|AgCl); this value is 320 mV more positive than that of unmodified cobaltocene, owing to the electron withdrawing nature of the carboxyl groups. Chemical reduction to the active Co^{II} form, 1,1′-dicarboxycobaltocene (M_{red}), was achieved with zinc dust. [3] Expression, purification, and quantification of BM3 and the heme domain of BM3 (hBM3) for use with the mediator were performed as described [22].

Absorption spectra were recorded to observe flavin reduction by the putative mediator. The flavins are the most readily reduced centers: FAD is rapidly reduced by soluble reductants (e.g., NADPH), and electron transfer between the two flavins is fast [23]. In

[2] See Supplementary material for details on the synthesis.

[3] Reduction was achieved by adding 500 mg of zinc dust to 15 mg of M_{ox} and 8 ml of degassed buffer (25 mM Tris hydroxymethylaminoethane–HCl, 25 mM potassium phosphate, 250 mM KCl, pH 8.0). This mixture was stirred under argon for 4 h and then filtered to remove the zinc dust. The result was a dark red solution (λ_{max} = 490 nm) of M_{red} solubilized in a buffer that could be readily used in an enzymatic reaction.

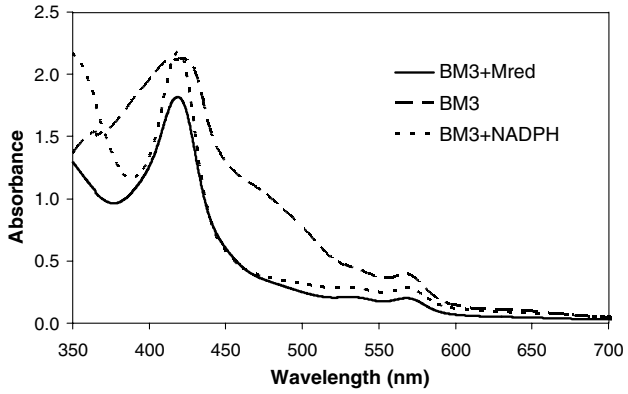

Fig. 2. BM3 absorption spectra (oxidized and reduced proteins): the shoulder between 440 and 490 nm attributable to oxidized flavins disappears upon reduction by NADPH and M_{red}.

Fig. 2, the shoulder between 440 and 490 nm is characteristic of oxidized flavins in BM3 [24]; this shoulder disappears upon reduction by NADPH. As can be seen from Fig. 2, adding M_{red} to a solution of BM3 results in a spectrum similar to that obtained after adding NADPH. This confirms flavin reduction by M_{red}, and suggests that M_{red} can indeed function in place of NADPH.

Addition of M_{red} to a solution of BM3 saturated with CO gave a peak at 448 nm (Fig. 3), characteristic of the Fe^{II}–CO species [6]. Notably, heme reduction by M_{red} occurs in the absence of substrate, unlike in the native system where electron transfer from flavins to the heme requires substrate at the active site [25]. Substrate displaces water from the heme pocket, converting Fe^{III} from low to high spin. This conversion is accompanied by a positive shift in the heme potential (>100 mV) that favors electron transfer from FMN to the heme. It appears that M_{red} reduces the heme directly, bypassing the reductase pathway. To test this, M_{red} was added to a CO-saturated solution of

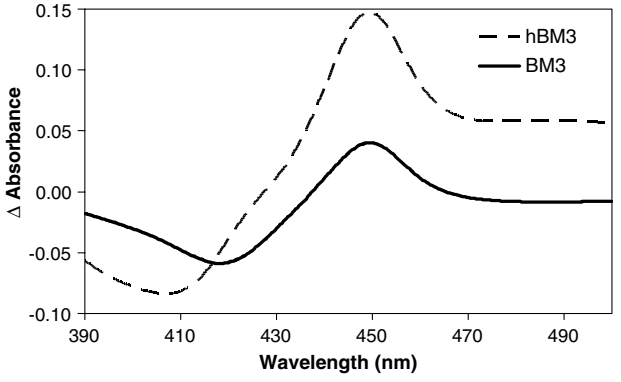

Fig. 3. Spectra of both holo (BM3) and heme domain (hBM3) BM3 in the presence of carbon monoxide and M_{red}: the peaks at 448 nm indicate heme reduction by M_{red} and formation of an Fe^{II}–CO complex.

Table 1
Rates and total turnovers for the electrochemical biocatalytic reactions

Enzyme	Mediator	Rate (nmol product/nmol enzyme/min)	Total turnover (nmols product/ nmols enzyme)
BM3	Cobaltocene cation	16.4 ± 0.6	224 ± 7
BM3	Cobalt(III) sepulchrate	37.8 ± 0.3	835 ± 7
hBM3	Cobaltocene cation	1.8 ± 0.5	58 ± 7
hBM3	Cobalt(III) sepulchrate	2.2 ± 0.1	76 ± 7

hBM3. As can be seen in Fig. 3, the Soret band at 448 nm shows that M_{red} reduced the heme iron. The hydrophobic substrate access channel [8] and the −2 charge on M_{red} make it unlikely that heme reduction occurs by direct interaction of the mediator with the active site. Apparently, M_{red} takes an alternative electron transfer pathway, thereby opening the way for catalysis without the reductase domain.

Enzyme activity assays were performed with lauric acid (K_m = 100 μM with BM3 [26]). Thirty-minute reactions in air were conducted with 1 μM BM3 or hBM3, 1 mM lauric acid, and 1 mM reductant (M_{red} or NADPH) in a final volume of 1 ml at room temperature. The reactions were quenched with five drops of concentrated HCl, and then samples were prepared for GC/MS analysis as described [22]. Negative controls lacking one component (substrate, mediator, or enzyme) were also carried out. The negative controls gave no product, while the NADPH and M_{red} samples for both BM3 and hBM3 yielded the expected hydroxylated compounds. The resulting product distribution of the M_{red} reactions, 36:28:36 for ω-1:ω-2:ω-3 hydroxy laurate, was similar to that of the NADPH-driven reaction [26]. To test if the observed products are a consequence of the peroxide shunt (from oxygen reduction to peroxide by M_{red}), reactions with M_{red} were also carried out in the presence of catalase. The resulting product profile was similar to that without catalase, [4] indicating that the reaction was metallocene-mediated: this was expected as previous work has shown that wild type BM3 does not have significant peroxide shunt activity [22].

Next, we developed an electrochemical system, where the soluble mediator provides electrons to the enzyme and is continuously regenerated at an electrode. [5] Co(sep) reactions were run in parallel with M_{red} reactions in order to compare the two mediators. The results in Table 1 reveal that the two mediator systems perform similarly under the conditions used. Notably, the rate

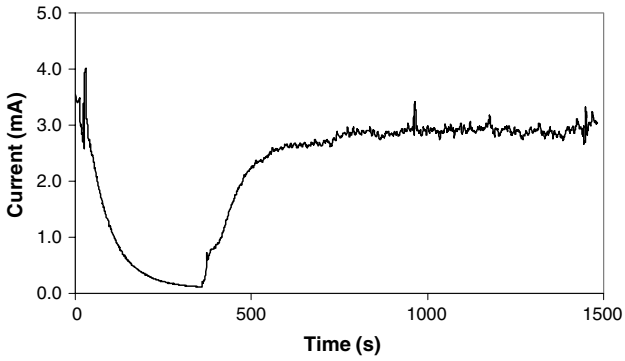

Fig. 4. Current vs. time for the electrochemical biocatalytic reaction: the initial decay represents anaerobic electrochemical reduction of M_{red}; this decay is followed by a spike in the current resulting from addition of oxygenated enzyme–substrate solution; a steady-state condition is established after 600 s (plateau region).

shown in Table 1 for Co(sep) with BM3 is less than that previously reported (38 vs. 110 min^{-1}); this probably results from continuously bubbling air into solution, which removes both dioxygen and the mediator from the reaction as they react with one another. The hBM3 results are intriguing: to our knowledge, this is the first report of mediated electrochemical catalysis with the heme domain. Although the reaction proceeds slowly, it nevertheless occurs and opens the possibility of performing the reaction with the heme domain alone, which could be advantageous given the propensity of the reductase to become inactivated through over-reduction [25].

The difference in rate between the mediated reactions and the native system can be partially understood by examining the current function. A typical current function from an M_{ox}–BM3 reaction is shown in Fig. 4. Initially, the reaction solution contains only M_{ox}. Applying a reducing potential results in a current that decays from an initial value of 4 mA to one that is near zero as M_{ox} is converted to M_{red}. Addition of the oxygenated enzyme–substrate solution followed by bubbling air into the reaction causes a spike and subsequent limiting current of 3 mA. From this limiting current, the coupling between total current passed by the electrode and total product formed can be calculated: [6] this yields a coupling efficiency of only 2% for the M_{red}–BM3 bioelectrochemical system. A similar calculation for hBM3 reactions yields only

[4] See Supplementary material for details and a GC trace of the catalase control reaction.

[5] See Supplementary material for details of the electrochemical reaction setup.

[6] Using the limiting current, the coupling between product formation and total charge passed can be calculated. From Table 1, 224 nmols of product are formed in the BM3 reaction. From Fig. 4, the total charge passed by the electrode during the biocatalytic reaction at the onset of the limiting current is 2.7 Coulombs. Applying the Faraday constant, the degree of coupling is calculated as the number of moles of product divided by half the number of moles of electrons (two electrons per product).

0.3%. These poor efficiencies attest to the acute sensitivity of cobaltocene to dioxygen [27]. Bubbling air into the reaction leads to rapid M_{red} oxidation, removing both dioxygen and the reduced mediator from the reaction as it becomes substrate limited; this undoubtedly contributes to the difference in rate between the mediated and native systems.

In summary, we have shown that the dicarboxy derivative of cobaltocene can function as an electrochemical mediator of P450 catalytic reactions. The main limitation of the M_{ox} system is its poor inherent coupling efficiency, owing largely to the sensitivity of M_{red} to dioxygen. Although the overall kinetics and turnover of Co(sep) with P450 are slightly better, M_{ox} can be modified more readily to optimize reaction parameters, providing a more versatile mediator.

Abbreviations

BM3	flavocytochrome P450 BM3
Co(sep)	cobalt(III) sepulchrate
M_{ox}	1,1′-dicarboxycobaltocenium hexafluorophosphate
M_{red}	1,1′-dicarboxycobaltocene
hBM3	heme domain P450 BM3

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2004.06.007.

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