Electron Transfer between the FMN and Heme Domains of Cytochrome P450BM-3

EFFECTS OF SUBSTRATE AND CO*

(Received for publication, October 2, 1996, and in revised form, December 5, 1996)

James T. Hazzard‡, Shanthi Govindaraju§, Thomas L. Poulos§, and Gordon Tollin‡

From the ‡Department of Biochemistry, University of Arizona, Tucson, Arizona 85721 and the §Department of Molecular Biology & Biochemistry and Physiology & Biophysics, University of California, Irvine, California 92697-3900

Cytochrome P450BM-3 has the P450 heme domain and FAD/FMN reductase domain linked together in a single polypeptide chain arranged as heme-FMN-FAD. In the accompanying article (Govindaraju, S., and Poulos, T. L. (1997) J. Biol. Chem. 272, 7915–7921), we have described the preparation and characterization of the various domains of cytochrome P450BM-3. One reason for undertaking this study was to provide simpler systems for studying intramolecular electron transfer reactions. In particular, the heme-FMN version of P450BM-3 that is missing the FAD domain should prove useful in studying the FMN-to-heme electron transfer reaction. This version of P450BM-3 has been designated truncated P450BM-3 or BM3t.

In this study we have used laser flash photolysis techniques to generate the reduced semiquinone of 5-deazariboflavin which in turn reduces the FMN to the semiquinone, FMN\(^{\bullet}\), at a rate constant of 6600 s\(^{-1}\), whereas the heme is not reduced by the 5-deazariboflavin radical. The reduction of the heme by FMN\(^{\bullet}\) does not proceed in the absence of carbon monoxide (CO), whereas in the presence of CO the FMN\(^{\bullet}\) to heme electron transfer rate constant is 18 s\(^{-1}\). If a fatty acid substrate is present, this rate constant increases to 250 s\(^{-1}\). Somewhat surprisingly, the rate of heme reduction also is dependent on [CO] which indicates that CO causes some change within the heme pocket and/or interaction between the heme and FMN domains that is required for intramolecular electron transfer.

Cytochrome P450BM-3 from Bacillus megaterium catalyzes the NADPH-dependent monooxygenation of fatty acid substrates (1). Unlike other P450s where the site of hydroxylation, the heme center, and the site of NADPH oxidation, the FAD center, reside on separate polypeptide chains, P450BM-3 has both activities on a single polypeptide chain of 119 kDa (2). As with eukaryotic microsomal P450s, the electron transfer flow in P450BM-3 is NADPH \(\rightarrow\) FAD \(\rightarrow\) heme. Unlike its eukaryotic counterparts, however, P450BM-3 provides an excellent system for studying intramolecular electron transfer (ET)\(^1\) from the flavin domain to the heme domain without the complications that arise from the protein-protein recognition and binding steps required for the eukaryotic P450 systems.

Recombinant expression of heme and diflavin reductase domains has provided simplified systems for detailed biophysical and biochemical studies. Sevrioukova and Peterson (3) and Sevrioukova et al. (4) have investigated the ET rates within the FAD/FMN reductase domain using NADPH and dithionite as reductants. In addition, Sevrioukova et al. (4) have measured the rate of heme reduction in holo-P450BM-3 using the physiological reductant, NADPH. It appears that ET from FMN to the heme is most efficient when the FMN is in the one-electron reduced semiquinone form. In the accompanying article (5) and in previous work (6) it has been shown that it also is possible to express and purify the individual FAD and FMN domains.

These studies demonstrate the modular architecture of P450BM-3 and also provide an opportunity to compare the properties of the domains to related proteins like flavodoxin (homologous to the FMN domain) and ferredoxin reductase (homologous to the FAD domain).

As described in the accompanying paper (5), we have prepared a version of P450BM-3 consisting of residues 1–664 that contains both the heme and the FMN domains (hereinafter referred to as a truncated BM3 or BM3t). BM3t is a simplified system for following the FMN \(\rightarrow\) heme ET reaction and provides the advantage of removing the spectral and kinetic ambiguities introduced by the presence of two flavin cofactors. In an attempt to directly measure the kinetics of intramolecular ET between the FMN and the heme, we have utilized the ability of free flavin semiquinones, generated by laser flash photolysis (7, 8), to act as in situ one-electron reductants. By this method, a brief laser pulse (<1 \(\mu\)s), in the presence of a sacrificial electron donor such as EDTA or semicarbazide, produces an exogenous flavin semiquinone which then acts as an efficient one-electron reductant of a redox protein. The optimal situation in this type of experiment can be achieved if initial reduction of BM3t occurs at the FMN center, rather than at the heme, with a rate sufficiently faster than any subsequent intramolecular ET reactions, since 1) the direction of electron flow is from FMN to the heme in the presence of either O\(_2\) or CO, 2) FMN is not readily reduced by NADPH (9), and 3) reduction of the FMN by exogenous flavin semiquinone will...
generate a one-electron reduced FMN species, which occurs in the holoenzyme during turnover prior to ET to the heme (4).

In the present study we have utilized 5-deazariboflavin semiquinone (dRFH) as the exogenous reductant, which has a quite negative midpoint potential (−630 mV) (10). This ensures that the initial bimolecular reduction of a redox protein is sufficiently fast (10⁸–10⁹ M⁻¹ s⁻¹ (8)) that any subsequent intramolecular ET reaction can be readily resolved both kinetically and spectrally. In view of the enzymatic function of P450BM-3, we have evaluated the effects of substrate (myristate) and CO binding on both the initial reduction reaction and the subsequent intramolecular ET between FMN and heme.

MATERIALS AND METHODS

BM3, was prepared and isolated as described in Govindaraj and Poulos (5). Steady-state photoreduction of BM3, (10 μM) was performed in a 100 mM phosphate buffer solution (pH 7.0) containing 5 μM deazariboflavin (dRF) and 5 mM semicarbazide as a sacrificial electron donor. The solution was bubbled with either oxygen-free argon or CO for at least 1 h prior to anaerobic addition of BM3t. Irradiation was carried out in 30-s illumination increments using a 30-watt tungsten illuminator. Difference spectra were collected with an OLIS-modified Cary 15 spectrophotometer using the fully oxidized BM3, as a reference spectrum.

Laser flash photolysis was carried out as described elsewhere (8). For all reactions, a 100 mM phosphate buffer solution (pH 7.0 containing 100 μM deazariboflavin and 2 mM semicarbazide was used. The solution (minus enzyme) was made anaeroic by bubbling for 1 h with either argon or CO-nitrogen mixtures which had been passed through a 1.5 × 100-cm column containing an oxygen removing catalyst (R3–11, Chemical Dynamics). The absence of O₂ was monitored by the amplitude and decay of the 5-dRFH transient signal obtained at 500 nm upon laser excitation of the reaction prior to addition of the enzyme. In order to maintain anaerobiosis, all aliquots of added protein (>5 μl) or substrate (0.5 μl) were subjected to a flow of the O₂-sparged gas prior to mixing with the bulk reaction solution (0.5 ml). The protein concentration was always larger than the concentration of 5-dRFH generated by the laser flash, so that pseudo-first order conditions applied and no more than a single electron could enter each protein molecule. Transient kinetic data were collected using a Tektronix TDS 410A digitizing oscilloscope and analyzed on a PC using KINFIT (OLIS). Kinetic traces corresponding to FMN reduction or reoxidation and heme reduction could be well fit with a single exponential equation.

RESULTS AND DISCUSSION

Redox Difference Spectra—In order to characterize the spectral species generated in the laser flash photolysis experiments, BM3t, in a solution containing dRF and the sacrificial donor semicarbazide, was reduced by white light irradiation for a series of fixed time intervals (~30 s) under either anaerobic or CO-saturated conditions. In the absence of CO and substrate (myristate), the reduced minus oxidized difference spectrum shown by the solid line in Fig. 1 was obtained. This spectrum, with a minimum at ~470 nm, is consistent with the conversion of the oxidized FMN prosthetic group of BM3, to the fully reduced species. The absence of significant positive absorption in the region from 580 to 610 nm indicates that there is little or no neutral FMN semiquinone present in this solution. Further irradiation of the sample resulted in the reduction of the oxidized heme (data not shown), despite the absence of substrate and CO. However, extensive illumination times were required to accomplish this, consistent with the expectation that the midpoint potential of the FMN is more positive than that of the heme.

When this procedure was performed in a CO-saturated buffer (~960 μM), the difference spectrum (dashed line) shown in Fig. 1 was obtained. This spectrum corresponds to CO-bound reduced heme (HCO) minus oxidized heme. Note that the contribution of the FMN to this difference spectrum is minimal, the major characteristic being the large increase in absorbance at 450 nm. Further irradiation of the sample resulted in a loss of absorbance in the region from 450 to 500 nm, indicating reduction of the oxidized FMN (not shown). It was also determined that exposure of the oxidized heme to CO, in the absence of any irradiation, did not alter the heme spectrum, indicating that on the time scale of this experiment there was no CO-induced autoreduction of the BM3, heme.

Time-resolved difference spectra, resulting from laser flash induced one-electron reduction of the enzyme, are also shown in Fig. 1. When a CO-saturated solution containing 5 μM BM3t, myristic acid (40 μM), dRF, and semicarbazide was illuminated using laser flash excitation at a wavelength equivalent to the absorption maximum of dRF (~395 nm), the semiquinone of the free flavin, dRFH, was rapidly (∼1 μs) generated, and subsequent one-electron reduction of BM3t occurred via a bi-molecular reaction (cf. Fig. 2A). It should be stressed that under these conditions of these experiments, [dRFH] ≪ [BM3t]₀ and, as in much as dRFH is a one-electron reductant, any given enzyme molecule was reduced by only 1 eq (7, 8). The initial rapid spectral changes generated by this process are shown by the filled circles in Fig. 1. These data were obtained from the magnitude of the transient signals obtained at 0.5 ms after the laser flash. The rationale for this is as follows. Upon flash irradiation of the reaction solution, a very rapid (<1 μs) increase in absorbance occurs, consistent with the formation of the deazariboflavin semiquinone (cf. Fig. 2A). This is followed by a slower change in absorbance consistent with reduction of
the enzyme and concomitant oxidation of the deazariboflavin semiquinone. At the concentration of enzyme used to obtain the transient redox difference spectra in Fig. 1, the reaction is complete in <0.5 ms. Thus, the signal amplitude at this point represents the initially reduced enzyme species. In the absence of CO, regardless of the presence or absence of substrate, this initial reduction product is stable for at least 300 ms after laser excitation (data not shown.) The initial reduction species has spectral properties similar to that obtained by steady-state photoreduction of BM3, in a CO-saturated buffer (data not shown). It should also be noted that if flash photolysis experiments are conducted in the absence of CO, only FMN reduction is observed on time scales up to 2 s after the laser flash, irrespective of the presence or absence of substrate (not shown).

**Kinetics of FMN and Heme Reduction**—The kinetics of the change in absorbance at 480 nm, corresponding to reduction of the BM3, FMN by dRFH, in a CO-saturated buffer and in the absence of substrate, is illustrated in Fig. 2A. The smooth line through the data represents the fit to a single exponential equation, giving a rate constant of 6600 s\(^{-1}\). Fig. 3 shows a plot of the values of \(k_{obs} \) versus \([BM3]_{ox}\) for the initial FMN reduction step for experiments conducted in both CO-free and CO-saturated buffers. A second order rate constant of \(7.8 \times 10^8\) M\(^{-1}\) s\(^{-1}\) is obtained which, within experimental error, is independent of the presence or absence of CO. It is interesting to compare these results with those obtained previously in this laboratory for the reduction of microsomal P450 reductase (13, 14). One-electron reduction of the microsomal reductase was shown to occur at the FAD cofactor with a second order rate constant of \(8 \times 10^7\) M\(^{-1}\) s\(^{-1}\), followed by a slow intramolecular ET
s^-1) to the oxidized FMN, forming the neutral semiquinone of FMN. Thus, the second order rate constant for reduction of the FMN in BM3t is an order of magnitude greater than that for FAD reduction in the microsomal reductase, suggesting a much larger degree of steric exposure of the FMN in the BM3t construct than either the FAD or the FMN cofactors in the microsomal reductase.

As noted above, in a flash experiment in the absence of CO we observed no heme reduction on time scales ≤2 s, whereas in a CO-saturated buffer formation of the CO-bound reduced heme was readily observed. This is shown in the kinetic traces at 480 nm, corresponding to FMN oxidation; ○, data obtained at 455 nm, corresponding to heme reduction and CO binding. The solid line represents a second order rate constant for CO binding having a value of 3.1 × 10^6 M^-1 s^-1. For all experiments, [BM3t] = 10 μM.

Furthermore, the rate constants at either wavelength are independent of enzyme concentration up to 40 μM (not shown). These results demonstrate that initial reduction of the enzyme, even in the presence of CO and/or substrate, occurs at the FMN site and that direct reduction of the heme group by dRFH is not a significant occurrence. In addition, in the absence of CO the reduced FMN is unable to transfer an electron to the heme on a time scale of ~2 s, whereas the presence of CO allows the FMN-to-heme ET reaction to occur.

The Effect of Substrate on Heme Reduction—If a substrate-free CO-saturated solution is titrated with myristate, there is a marked increase in the value of k_obs for heme reduction, as shown in the kinetic transient obtained at 460 nm in Fig. 2C (compare with Fig. 2B). At [myristate] = 35 μM, the rate constant obtained from the single exponential fit to the data in Fig. 2C has a value of 250 s^-1. The dependence of k_obs on [myristate] is shown in Fig. 4A. The data clearly follow a hyperbolic curve, with a limiting value of 350 s^-1 at infinite substrate concentration. Substrate binding is not a significant occurrence. In addition, in the absence of CO the reduced FMN is unable to transfer an electron to the heme on a time scale of ~2 s, whereas the presence of CO allows the FMN-to-heme ET reaction to occur.

**Fig. 4.** The dependence of k_obs values obtained upon laser flash photolysis on CO and myristate concentrations. A, titration of CO-saturated reaction solution with myristate. The solid line represents the best fit to the data using an equation corresponding to a two-step mechanism, from which we calculate K_M = 6.8 × 10^-6 M for myristate binding and k_0 = 350 s^-1 at infinite substrate concentration. B, dependence on [CO] in the reaction buffer, obtained by increasing the percentage of CO in a mixture with N2. ○, data obtained at 480 nm, corresponding to FMN oxidation; △, data obtained at 455 nm, corresponding to heme reduction and CO binding. The solid line represents a second order rate constant for CO binding having a value of 3.1 × 10^6 M^-1 s^-1. For all experiments, [BM3t] = 10 μM.

**Fig. 5.** Transient kinetics obtained at 450 nm (A) and 480 nm (B) following BM3t reduction. The buffer conditions were as in Fig. 2, except [myristate] = 45 μM and [CO] = 0.825 mM, corresponding to 85% saturation. The smooth lines through the transients represent a single exponential fit, giving rate constants of 270 s^-1 (A) and 260 s^-1 (B).

Intramolecular Electron Transfer in Cytochrome P450BM-3
strongly favored at pH 8.0 and high ionic strength and the $k_d$ = 0.5 μM spectral titration was carried out at pH 8.0 in 0.5 M buffer (15) while in the present study we used a 0.1 M pH 7 buffer. However, if we repeat the spectral titration experiment as in Ref. 15 except using the same buffer used for the flash photolysis experiment, a $k_d$ ~ 5 μM is obtained, in excellent agreement with 6.8 μM estimated in the present study.

Figs. 4B and 5 illustrate that the kinetics corresponding to heme reduction and CO binding are identical to those corresponding to FMN oxidation. Fig. 5 shows the absorbance changes at 455 nm (heme) and 480 nm (FMN) on a 20-msec time scale, following reduction of the enzyme by dRPH in a solution containing 45 μM myristate (~7-fold larger than the $k_d$ value given above) and 0.8 mM CO (85% saturation). The rate constant obtained from both data sets has a value of 275 s⁻¹. Fig. 4B shows a plot of $k_{obs}$ versus [CO] for the formation of the HCO complex, in the presence of 45 μM substrate. The close agreement between the rate constants obtained at the two wavelengths clearly shows that the reaction of the heme and CO binding is concomitant with FMN reoxidation over the accessible range of [CO]. Furthermore, there is a linear dependence on [CO], indicating that the reaction is strictly bimolecular, with an apparent second order rate constant, $k_{2}$ = 3.1 × 10⁵ M⁻¹ s⁻¹. These data suggest that the rate-limiting reaction in the overall mechanism is CO binding.

Conclusions—In this study we have found that dRPH rapidly reduces the FMN in BM3, to the anionic semiquinone, which is thought to be the active species in the steady-state reaction of the holoenzyme of P450BM-3 (4), without significant dRPH reduction of the heme center. In the presence of CO, this is followed by a slower ET reaction from FMN. However, if we repeat the spectral titration experiment as in Ref. 15 except using the same buffer used for the flash photolysis experiment, a $k_d$ ~ 5 μM is obtained, in excellent agreement with 6.8 μM estimated in the present study.

The lack of $k_d$ reduction by FMN⁺, in the absence of CO or substrate, is probably due to the iron being in the high spin state with a redox potential lower than that of FMN⁺, so that intramolecular ET from FMN⁺ to Hox is thermodynamically unfavorable. The [CO] dependence and no build-up of (FMN−Hox) complex is in agreement with Scheme 1 if $k_{1} < k_{-1}$ and $k_{2} > k_{-2}$. This latter assumption is valid since $k_{d}$ = 3.9 × 10⁶ M⁻¹ s⁻¹ and $k_{d}$/$k_{-d}$ = 5.1 × 10⁵ M⁻¹ s⁻¹. Since [CO] ≫ [FMN Hox], CO binding will be a pseudo-first order process giving linear plots of $k_{obs}$ versus [CO], which is observed (cf. Fig. 4B). The main drawback to Scheme 1 is the requirement that $k_{1} < k_{-1}$. In fact, we expect just the opposite since with substrate and CO present, the heme redox potential should be higher than FMN⁺, thereby favoring the formation of FMN-Hox, and thus the equilibrium constant, $k_{d}/k_{-d}$, should be >> 1.0.

Alternatively, we consider Scheme 2 in which there is a pre-equilibration step between CO and (FMN−Hox)S. As noted above, in the absence of both substrate and CO, Hox is not reduced by FMN⁺. However, when CO is added, ET to Hox occurs. This effect of CO is likely due to an increase in the heme redox potential (16). This implies the presence of CO leads to some structural perturbation within the heme pocket. Presumably, this does not involve formation of an Fe-CO bond, since the iron is still oxidized (Fe³⁺) and CO does not affect the spectral properties of BM3, in the ferric heme state. To account for the [CO] dependence and the lack of (FMN Hox)S build-up according to Scheme 2, $k_{2} >> k_{1}$ and the rate-limiting step is conversion of the heme environment by CO to a form suitable for accepting an electron from FMN⁺. Precisely what the nature of this perturbation might be remains unknown. However, we do know that the P450BM-3 heme domain exists in at least two different conformational states. The substrate-free crystal structure shows that the substrate access channel adopts an open conformation, whereas the substrate-bound crystal structure (17) shows that the access channel closes. This requires substantial movement of several elements of secondary structure. In addition, Phe-87, which is directly adjacent to the O₂ binding site, is perpendicular to the heme without substrate, but parallel to the heme when substrate binds. Consistent with recent NMR studies (18), the crystal structure also shows that the substrate is too far from the iron, ~7.8 Å, for hydroxylation. Additional NMR studies have shown that the substrate moves much closer to the heme iron upon reduction of the iron from Fe³⁺ to Fe²⁺. Therefore, P450BM-3 appears to exist in multiple conformational states, some requiring rather large global changes and others requiring alterations within the heme pocket. As a result, it is not unreasonable to consider that a relatively hydrophilic molecule like CO can enter the large substrate pocket, even when the heme is in the ferric state, and induce changes that favor reduction of the heme. Precisely how remains an interesting question but could involve local perturbations within the heme pocket or, possibly, adjustments between the FMN and heme domains. The methods employed in this study together with mutagenesis and additional crystal structure work should help to answer some of these questions.

REFERENCES
1. Narhi, L. O., and Fulco, A. J. (1986) J. Biol. Chem. 261, 7160–7169
2. Narhi, L. O., and Fulco, A. J. (1987) J. Biol. Chem. 262, 6683–6690
3. Servioukova, I. F. and Peterson, J. A. (1995) Biochimie (Paris) 77, 562–572
4. Servioukova, I. F., Shaffer, C., Ballou, D. P., and Peterson, J. A. (1996) Biochemistry 35, 7055–7068
5. Govindaraj, S., and Poulos, T. L. (1997) J. Biol. Chem. 272, 7915–7921
6. Servioukova, I. F., Truan, G., and Peterson, J. A. (1996) Biochemistry 35, 7528–7535
7. Tollin, G., and Hazzard, J. T. (1991) Arch. Biochem. Biophys. 287, 1–7
8. Tollin, G., Hurley, J. K., Hazzard, J. T., and Meyer, T. E. (1993) Biochim. Biophys. Acta 1210, 259–279
9. Govindaraj, S., Li, H. Y., and Poulos, T. L. (1994) Biochim. Biophys. Res. Commun. 203, 1745–1749
10. Blankenhorn, G. (1976) Eur. J. Biochem. 67, 67–80
11. Edmondson, D. E., and Tollin, G. (1983) Top. Curr. Chem. 108, 109–138
12. Servioukova, I. F., and Peterson, J. A. (1996) Arch. Biochem. Biophys. 317, 397–404
13. Bhattacharyya, S. K., Lipka, J. J., Waskell, L., and Tollin, G. (1991) Biochemistry 30, 759–765
14. Bhattacharyya, A. K., Hurley, J. K., Tollin, G., and Waskell, L. (1994) Arch. Biochem. Biophys. 310, 318–324
15. Govindaraj, S., and Poulos, T. L. (1995) Biochemistry 34, 11221–11226
16. Servon, J. A., and Boddupalli, S. S. (1992) Arch. Biochem. Biophys. 294, 654–661
17. Li, H. Y., and Poulos, T. L. (1996) Nat. Struct. Biol. 4, 140–146
18. Modi, S., Primrose, W. U., Boyle, J. M. B., Gibson, C. F., Lian, L. Y., and Roberts, G. C. (1995) Biochemistry 34, 8982–8988