Giese, B., Gröninger, K. S., Witzel, T., Korth, H.-G., & Sustmann, R. (1987) *Angew. Chem., Int. Ed. Engl. 26*, 233-234.

Haslam, E. (1974) *The Shikimate Pathway*, Wiley, New York.

Keck, G. E., Enholm, E. J., Yates, J. B., & Wiley, M. R. (1985) *Tetrahedron 41*, 4079-4094.

Lambert, J. M., Boocock, M. R., & Coggins, J. R. (1985) *Biochem. J. 226*, 817-829.

Maitra, U. S., & Sprinson, D. B. (1978) *J. Biol. Chem. 253*, 5426-5430.

Meloche, H. P., & Wood, W. A. (1964) *J. Biol. Chem. 239*, 3505-3510.

Messner, B., Eggerer, H., Cornforth, J. W., & Mallaby, R. (1975) *Eur. J. Biochem. 53*, 255-264.

Ohrui, H., Horiki, H., Kishi, H., & Meguro, H. (1983) *Agric. Biol. Chem. 47*, 1101-1106.

Parry, R. J., & Askonas, L. J. (1985) *J. Am. Chem. Soc. 107*, 1417-1418.

Rotenberg, S. L., & Sprinson, D. B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1669-1672.

Rotenberg, S. L., & Sprinson, D. B. (1978) *J. Biol. Chem. 253*, 2210-2215.

Scharf, K. H., Zenk, M. H., Onderka, D. K., Carroll, M., & Floss, H. G. (1971) *J. Chem. Soc., Chem Commun.*, 765-766.

Schwab, J. M., Klassen, J. B., & Habib, A. (1986) *J. Chem. Soc., Chem. Commun.*, 357-358.

Sedgwick, B., Morris, C., & French, S. J. (1978) *J. Chem. Soc., Chem. Commun.*, 193-194.

Srinivasan, P. R., Rothschild, J., & Sprinson, D. B. (1963) *J. Biol. Chem. 238*, 3176-3182.

Tejima, S., & Fletcher, H. G., Jr. (1963) *J. Org. Chem. 28*, 2999-3004.

Turner, M. J., Smith, B. W., & Haslam, E. (1975) *J. Chem. Soc., Perkin Trans. 1*, 52-55.

Weiss, U., & Edwards, J. M. (1980) *The Biosynthesis of Aromatic Compounds*, Wiley, New York.

Whistler, R. L., & Anisuzzaman, A. K. M. (1980) *Methods Carbohydr. Chem. 8*, 227-231.

Willarden, P., & Eggerer, H. (1975) *Eur. J. Biochem. 54*, 247-252.

Effect of the Distal Histidine Modification (Cyanation) of Myoglobin on the Ligand Binding Kinetics and the Heme Environmental Structures

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ABSTRACT: The kinetics of carbon monoxide (CO) binding to myoglobin (Mb) modified at the distal histidine (His) by cyanogen bromide (BrCN) has been studied. The CO association and dissociation rates of BrCN-modified Mb were obtained as 1.8×10^3 M^{-1} s^{-1} and 0.13 s^{-1}, respectively (20 °C and pH 7.0). Thermodynamic parameters were obtained as well. These values are notable, compared with those for other hemoproteins, the slowest association and the fastest dissociation rates among various hemoproteins examined so far. On the basis of the available structural data obtained from the absorption, ^1H NMR, and IR spectral measurements, these unique kinetic and thermodynamic properties were reasonably explained in terms of the steric restriction at the modified distal side.

We previously reported that the distal histidine (His E7) of sperm whale myoglobin (Mb) is specifically modified by reaction with an equimolar amount of cyanogen bromide (BrCN) (Shiro & Morishima, 1984), where the imidazolyl N-H of the distal His is displaced by the substituted N-CN. This distal His modification caused drastic ^1H NMR and absorption spectral changes of aquometMb, which suggested that the heme evnironmental structure of the BrCN-modified Mb (BrCN-Mb) is very different from that of native Mb. Quite different features of the hyperfine-shifted ^2H NMR resonances for meso-deuterated porphyrin contaning native and BrCN-modified Mb's in the aquomet state indicated that the water molecule coordinated to the heme iron at the sixth site is expelled upon BrCN modification (Morishima et al., 1985). It was also noted that ferric BrCN-Mb cannot combine with external ligands such as CN^- and imidazole which potentially bind to native Mb. These findings suggest that BrCN-Mb has unique ligand binding properties, due to the substantial structural changes in the heme distal site.

The importance of the distal His in controlling the heme iron reactivities of Mb and hemoglobin (Hb) has long been suggested on the basis of several biochemical and physicochemical investigations. In this connection, structural factors such as steric restriction and hydrogen-bonding interaction have been pointed out on the basis of X-ray and neutron diffraction studies of CO and O_2 complexes of Mb and Hb (Norvell et al., 1975; Baldwin, 1980; Phillips, 1980; Phillips & Schoenborn, 1981; Hanson & Schoenborn, 1981; Kuriyan et al., 1986). The distal His effects on ligand binding are pronouncedly manifested in the kinetic properties of mutant Mb and hB, in which the distal His is replaced by other amino acid residues (Giacometti et al., 1980; Parkhurst et al., 1980;

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Amiconi et al, 1972; Wittenberg et al., 1972). For example, for Hb Zürich, in which the distal His of the β subunit is replaced by an arginine, the association rate and the affinity of the ligand are relatively large (Giacometti et al., 1980), compared with those for normal Hb. In relation to these mutant hemoproteins, the unique features of ligand binding properties for BrCN-Mb should be examined in more detail.

We report here on the kinetics of CO binding to BrCN-Mb and some spectroscopic properties of the complexes. Compared with those for other hemoproteins, its unique ligand binding properties are explored and discussed in relation to the structural specificities of BrCN-Mb in the heme distal side.

MATERIALS AND METHODS

Sperm whale myoglobin (type II) was obtained from Sigma. Since sperm whale myoglobin from Sigma contains its ferrous form as a minor component, a solution of this Mb was oxidized once by a 2–3-fold excess of potassium ferricyanide and was used after gel filtration on a Sephadex G-25 column equilibrated with 0.1 M KPB, pH 7. Cyanogen bromide (BrCN) was purchased from Wako Pure Chemical, Ltd. Modification of metMb by BrCN was carried out by the method described previously (Shiro & Morishima, 1984; Jajczay, 1970), that is, by simple addition of an almost equimolar amount of BrCN to a metMb solution at pH 7, and the modification was monitored by its UV–visible spectrum. Specific cyanation of the distal His of aquometMb by BrCN and the homogeneity of the BrCN-Mb sample were checked ^{15}N and ^{13}C NMR spectroscopy of $BrC^{15}N$-Mb and $Br^{13}CN$-Mb, respectively (Adachi and Morishima, submitted for publication). The ^{15}N and ^{13}C NMR spectra of $BrC^{15}N$-Mb and $Br^{13}CN$-Mb showed a single peak at −301 ppm from external $^{15}NO_3^-$ and at 129 ppm from $[^{13}C]$TMS, respectively. These resonance positions were identical with those observed for the reaction mixture of labeled BrCN and histidine in the same buffer solution, which has been shown to form an N-cyanated imidazolyl group (Kanaya & Yanagawa, 1986). The UV–visible spectrum of this modified metMb slowly changes to that of the native form in a few days, and we used the modified sample soon after the modification. The protein concentrations were determined spectrophotometrically with extinction coefficients of 164 mM^{-1} cm^{-1} at 409 nm for native aquometMb and of 103 mM^{-1} cm^{-1} at 397 nm for BrCN-metMb. To prepare the CO complex of native and BrCN-modified Mb, 5–15 μM metMb solutions were equilibrated with nitrogen, and then a bit of dithionite was added. The reduced Mb solutions were then equilibrated with CO gas at 0.2, 0.4, 0.6, 0.8, and 1.0 atm. The nitric oxide complex was prepared by addition of a small amount of $NaNO_2$ and $Na_2S_2O_4$ to the native and BrCN-modified metMb solutions under anaerobic conditions. All sample solutions for the spectral and kinetic measurements were prepared in 0.1 M phosphate buffer at pH 7. Addition of O_2 to the reduced BrCN-Mb affords an extraordinarily stable ferrous low-spin complex of Mb, but this complex did not soon change to the ferrous high-spin complex even after photoexitation by a dye laser and is too stable to be autoxidized or replaced by the CO. We assume this complex is not the O_2 form of BrCN-Mb but a complex formed by the reaction of imidazolyl N–CN and O_2.

CO association and dissociation rates of Mb's were measured by a laser flash photolysis method with an Otsuka Electronics (Photal) RA401. Because of the instability of the BrCN-Mb·CO complex (Shiro & Morishima, 1984), in which decyanation of the modified site immediately occurs, the measurement was carried out within 5 min after complex preparation.

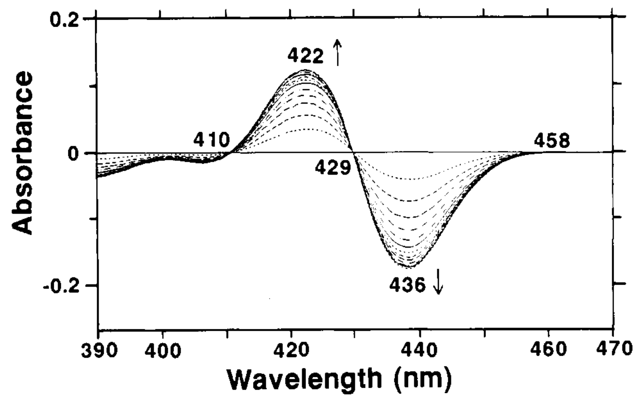

FIGURE 1: Rapid time difference spectra of the recombination of BrCN-Mb with CO in 0.1 M KPB, pH 7, at 20 °C, in the presence of 1 mM CO, measured within 5 min after the formation of the CO complex. BrCN-Mb concentration, 10 μM. The difference spectra were obtained with a 100-ms interval, the spectrum recorded 5 ms after photolysis being used as a reference.

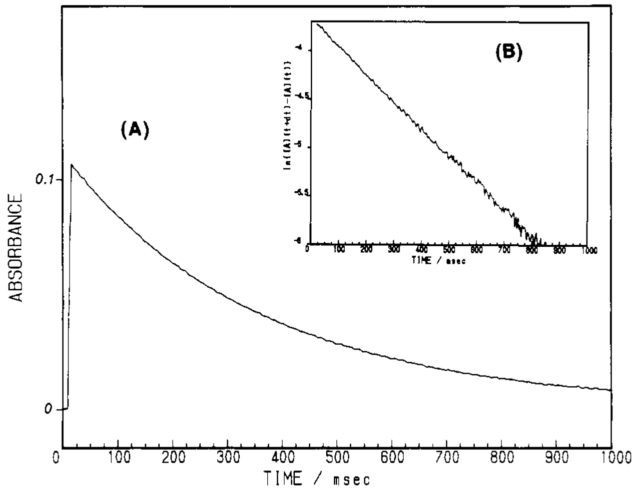

FIGURE 2: (A) Absorption kinetics of BrCN-Mb·CO monitored at 436 nm. BrCN-Mb concentration, 6 μM. Conditions are the same as in Figure 1. (B) Guggenheim plot of the kinetics of BrCN-Mb·CO.

The ESR spectra of the NO complex of BrCN-Mb were carried out at X-band (9.35 GHz) microwave frequency by use of a Varian cavity with a home-built spectrometer with 100-kHz modulation and at 77 K.

RESULTS

CO Binding to BrCN-Modified Myoglobin. When the CO complex of BrCN-Mb is photodissociated at 20 °C in the presence of 1 mM CO at pH 7, within 5 min after the formation of the CO complex, the rapid time difference spectra (Figure 1) showed uniform changes with isosbestic points at 410, 429, and 458 nm, throughout the course of recombination. The resultant difference spectrum is consistent with that between deoxy and CO forms of ferrous BrCN-Mb. When the CO recombination was followed at 436 nm within 5 min after complex formation, a characteristic monophasic absorbance change was obtained (Figure 2A), and the Guggenheim plot of this absorbance change (Figure 2B) is linear, which shows kinetically the homogeneity of BrCN-Mb·CO. After more than 5 min, BrCN-Mb·CO reverts in time to native Mb·CO as observed in a previous study (Shiro & Morishima, 1984), and the absorbance change after photodissociation of CO shows a biphasic curve. Therefore, we used kinetic data measured within 5 min after the formation of the CO complex of modified Mb for further analysis. The apparent first-order rate constant (k_{app}) exhibited a linear dependence on the CO

Table I: Kinetic Constants for CO Binding to Various Hemoproteins

	k_{on} ($\times10^{-5}$ M^{-1} s^{-1})	k_{off} (s^{-1})	K ($\times10^{-7}$ M^{-1})	ref
BrCN-modified Mb	0.018	0.13	0.0014	this work
sperm whale Mb	5.0	0.015	3.3	*a*
horse heart Mb	5.0	0.017	2.9	*a*
isolated α chain	40	0.013	30.8	*a*
isolated β chain	45	0.008	56.3	*a*
glycera Hb	270	0.042	64.0	*b*
Hb Thummii	270	0.095	28.4	*c*
leghemoglobin	127	0.016	79.0	*d*
β Zürich	220			*e*

[a] Antonini and Brunori, 1971. [b] Parkhurst et al., 1980. [c] Amiconi et al., 1972. [d] Wittenberg et al., 1972. [e] Giacometti et al., 1980.

Table II: Thermodynamic Constants for CO Binding to Native and BrCN-Modified Myoglobins

	ΔE_{on} (kcal mol^{-1})	ΔE_{off} (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
BrCN-Mb	12.5	17.4	−4.9	3
native Mb	4.1	25.0[b]	−20.9[a]	−35[a]

[a] Data from Rudolph et al. (1972). [b] Calculated from our data (ΔE_{on}) and those of Rudolph et al. (1972).

concentration as expected on the basis of the following simple reaction scheme:

$$\text{BrCN-Mb} + \text{CO} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{BrCN-Mb·CO} \qquad (1)$$

To obtain the pseudo-first-order rate constants, the absorbance changes at 436 nm for various amounts of CO were analyzed for this reaction. The kinetic parameters [k_{on}, k_{off}, and K ($=k_{on}/k_{off}$)] at 20 °C thus obtained are compiled in Table I, together with those for many other hemoproteins. Inspection of this table shows that BrCN-Mb is characterized by the low-affinity, slow association and fast dissociation rates of CO.

In order to gain further insight into the CO binding feature of BrCN-Mb, the thermodynamic parameters for this reaction were obtained by examining this reaction at various temperatures. From the resultant Arrhenius plot, the activation energies (ΔE_{on} and ΔE_{off}) for the CO association and dissociation reactions of BrCN-Mb were estimated as 12.5 and 17.4 kcal/mol, respectively. Furthermore, from the van't Hoff plot of K, the enthalpy (ΔH) and entropy (ΔS) changes were also obtained. These thermodynamic data are listed in Table II, together with those for native Mb. It is worth noting in this table that the ΔE_{on} and ΔS values for the CO association reaction in BrCN-Mb are larger than those in native Mb and are apparently unusual compared with those of other hemoproteins. The unique kinetic properties for BrCN-Mb obtained here will be discussed below in relation to its characteristic structure in the heme environment.

NO Complex of BrCN-Modified Myoglobin. Deoxy-BrCN-Mb can also slowly combine with NO to produce its NO complex. The UV–visible spectra of the NO complex are compared between native and BrCN-modified Mb in Figure 3. In Figure 4 is shown the ESR spectrum of the BrCN-Mb·NO complex. A triplet signal arising from ^{14}NO is not further split by the superhyperfine interaction with the iron-bound proximal His. This spectral feature has frequently been encountered for hemoproteins such as hemoglobin α chain bound with inositol hexaphosphate (Maxwell & Caughey, 1976; Szabo & Perutz, 1976), in which the Fe–proximal His bond is ruptured or weakened. We examined in more detail the ESR spectrum of the BrCN-Mb·NO complex with different time intervals after complex preparation. The broad ESR signal characteristic of a six-coordinate NO complex, which is contaminated with a five-coordinate complex signal (triplet signal), was obtained 2 min after sample preparation. The spectrum taken after 5 min was composed of a major component of the triplet signal and a minor one with broad six-coordinate-type signal. This six-coordinate-type signal decreased in time, followed by an increase of a new six-coordinate-type of broad signal at the expense of a five-coordinate-type triplet signal. These spectral changes could be explained as follows. Six- and five-coordinate BrCN-Mb·NO complexes were formed at first, and then six-coordinate complex was converted to five-coordinate complex, followed by formation of native Mb·NO complex due to back-reaction of BrCN-Mb to native Mb.

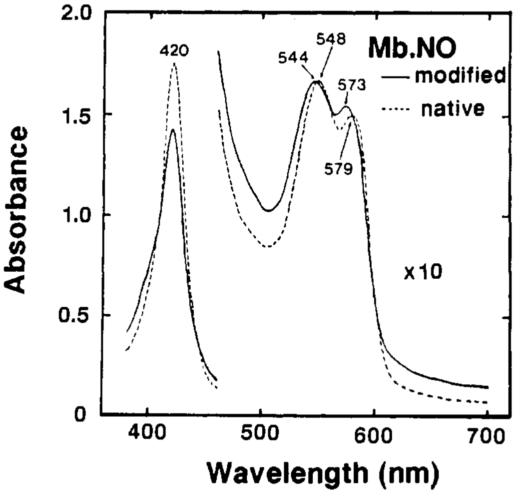

FIGURE 3: Optical absorption spectra of NO complexes of BrCN-Mb and native Mb in 0.1 M KPB, pH 7, at 20 °C.

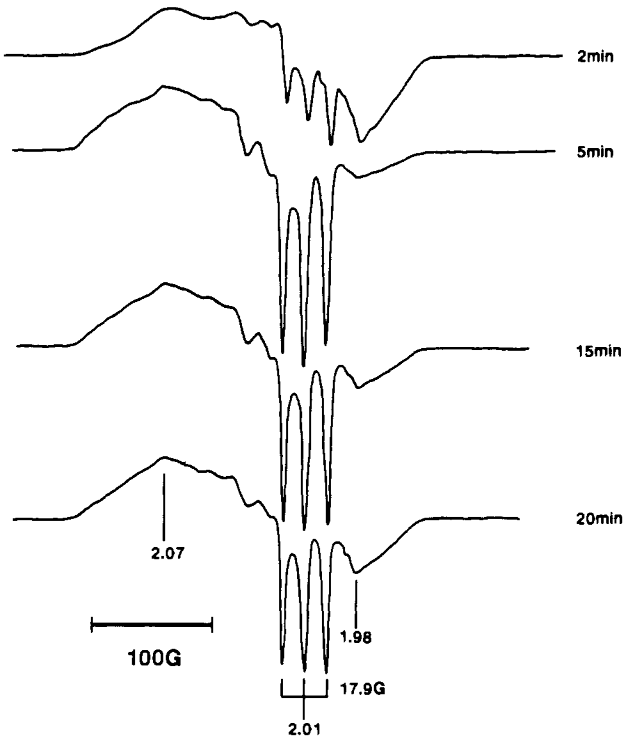

FIGURE 4: ESR time course spectra of the NO complex of BrCN-Mb in 0.1 M KPB, pH 7, at 77 K.

With this NO form of BrCN-Mb, we failed to measure the ligand binding kinetic constants, because the NO binding to BrCN-Mb was accompanied by the fast back-reaction to the native form.

DISCUSSION

Inspection of Table I shows that BrCN-modified Mb has very unusual CO binding properties, as compared with those for other hemoglobins and myoglobins. BrCN-Mb has the slowest binding rate and the lowest affinity for CO among the hemoproteins listed in Table I. Its unusual properties are also seen in the thermodynamic parameters (ΔE_{on}, ΔE_{off}, ΔH, and ΔS; Table II) for the CO binding reaction. It is apparent that CO has to go through an extremely high potential barrier to combine with BrCN-Mb, and the resultant BrCN-Mb·CO complex is relatively unstable, eventually resulting in a decyanation reaction to reproduce native Mb·CO. It is surprising that the modification of a single amino acid residue, the distal His, causes such a dramatic change in the ligand binding properties of Mb, which has never been experienced by the mutant Mb and Hb with amino acid replacement at the distal His side (Mins et al., 1983). With BrCN-Mb, we now have the opportunity to relate the ligand binding properties and the structural characteristics in the heme distal site.

Most investigators agree that at room temperature ligand binding to myoglobin can be described by a linear mechanism involving two or three consecutive reaction processes (Austin et al., 1975; Gibson et al., 1986; Jongeward et al., 1988). This scheme can be written as

$$S \underset{k_{MS}}{\overset{k_{SM}}{\rightleftharpoons}} M \underset{k_{BM}}{\overset{k_{MB}}{\rightleftharpoons}} B \underset{k_{AB}}{\overset{k_{BA}}{\rightleftharpoons}} A \qquad (2)$$

In eq 2, A denotes the state where the ligand is covalently bound to the heme iron, B where the ligand resides in the heme pocket, M where it migrates through the protein matrix, and S where it is in the solvent. In general, all rate constants shown in eq 2 are needed to fully characterize binding and dissociation processes. However, if the solvent process S is much faster than the initial rebinding process [as is the case for CO binding to Mb (Doster et al., 1982; Gibson et al., 1986)], the steady-state dissociation yield approaches unity, and the association rate is

$$k' = k_{BA}K_{SB} \qquad (3)$$

where K_{SB} is the equilibrium constant for state B formation ($K_{SB} = k_{MB}k_{SM}/k_{BM}k_{MS}$). Equation 3 indicates that the ligand in the solvent is in equilibrium with state B, and iron–ligand bond formation limits the overall association reaction. For CO binding to both native and BrCN-Mb, the bimolecular association rate constant becomes proportional to k_{BA}. However, since $k_{BM} \gg k_{BA}$, little or no geminate recombination can be observed in laser photolysis experiments at room temperature and in oridinary solvents, and it is very difficult to define the absolute value of k_{BA}. The uncertainties in k_{BA} and K_{SB} for CO binding at room temperature make it difficult to determine the cause of the much smaller overall association rate constants for CO binding to BrCN-Mb compared to that observed for native Mb.

Frauenfelder and co-workers (Doster et al., 1982; Dlott et al., 1983; Stetzkowski et al., 1985) reported the k_{BA} and K_{SB} [$P_B(c,T)$ in their nomenclature] values for CO binding to native sperm whale Mb (Mb), β chains of human hemoglobin (β^A) and hemoglobin Zürich [β63(E7)His → Arg] (β^{ZH}), and soybean leghemoglobin (Lb) by laser flash photolysis at temperatures from 10 to 320 K and in a glycerol–water mixture. For Hb Zürich, where His E7 in the β chain is replaced by Arg, and Lb, whose D-helix is absent, their cavity on the distal side of the heme is enlarged (Tucker et al., 1978; Ollis et al., 1983) and have much higher association rates for CO. In these papers, K_{SB} was shown to be nearly the same for all proteins studied except for Lb, whose K_{SB} shows little dependence on the solvent, and this was explained by access of the solvent to the heme pocket, while, in contrast, k_{BA} ranged about a factor of 100. So, they consequently concluded that the barrier between A and B dominates the control of the overall association rate and the protein structure of the distal side of the heme mainly affects k_{BA}, while K_{SB} does not seem to be used for control.

In the case of BrCN-Mb, our previous spectroscopic study (Shiro & Morishima, 1984) revealed that the heme environment of BrCN-Mb is so much different from that of native Mb in any iron oxidation/spin states, while the proximal structures in deoxy and CO forms are essentially unaltered upon the BrCN modification, as inferred from the NMR spectral change of the exchangeable proximal N_1H proton signal for the deoxy form and an absorption spectral change of the CO derivatives. It is thus likely that the effect of the heme proximal side on the functional difference in the CO binding of native and BrCN-modified Mb's is not important. By contrast, the distal-side structure of BrCN-Mb appears to play a crucial role in the ligand binding reaction. Modification of the distal histidyl imidazolyl NH to N–CN could exert steric constrains and prevent hydrogen bonding at the distal side. In fact, in the ferric state of BrCN-Mb, ligands such as CN^-, imidazole, and H_2O cannot bind to the heme iron. So, k_{BA} would be mainly affected by BrCN modification and be much smaller than that for native Mb. For BrCN-Mb, steric constraints introduced by the distal His modification sterically restrict access of CO to the heme iron sixth coordination position, resulting in its slow association rate. A large ΔE_{on} value also indicates some steric barrier toward the CO binding to heme iron.

It is also worthy noting in the CO combination reaction of BrCN-Mb that the entropy change ($\Delta S = 3$ cal K^{-1} mol^{-1}) is substantially large, compared with that for native Mb ($\Delta S = -35$ cal K^{-1} mol^{-1}). This could be due to the steric interaction of the modified distal His with the iron-bound CO, accompanied by protein conformational changes in the heme environment. In fact, upon BrCN modification, the distal residue Val E11 of Mb·CO moves farther away from the heme plane by 0.3 Å as visualized by the Val E11 methyl proton NMR (Shiro & Morishima, 1984). Upon BrCN modification, the off-axis configuration of the coordinated CO is more favored, as manifested in the IR spectral change of the coordinated CO stretching from 1944 to 1967 cm^{-1} (Shiro & Morishima, 1984). This IR shift is suggestive of destablilization of the CO coordination in BrCN-Mb, which appears to be consistent with the observed fast CO dissociation constant for BrCN-Mb·CO ($k_{off} = 0.13$ s^{-1}), compared with that for Mb·CO ($k_{off} = 0.015$ s^{-1}) at 20 °C and pH 7.0.

In the present kinetic and previous spectroscopic studies on the carbon monoxide complex of BrCN-Mb, the effect of distal His on ligand binding to the heme is directly explored.

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Registry No. CO, 630-08-0; NO, 10102-43-9; heme, 14875-96-8.

REFERENCES

Amiconi, G., Antonini, E., Brunori, M., Formaneck, H., & Huber, R. (1972) *Eur. J. Biochem. 31*, 52–58.

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, I. C. (1975) *Biochemistry 14*, 5355–5373.

Biochemistry **1989**, *28*, 7586-7592

Baldwin, J. M (1980) *J. Mol. Biol. 136*, 103-128.

Dlott, D. D., Frauenfelder, H., Langer, P., Roder, H., & DiIorio, E. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6239-6243.

Doster, W., Beece, D., Bowne, S. F., DiIorio, E. E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K. H., & Yue, K. T. (1982) *Biochemistry 21*, 4831-4839.

Giacometti, G. M., Brunori, M., Antonini, E., DiIorio, E. E., & Winterhalter, K. H. (1980) *J. Biol. Chem. 255*, 6160-6165.

Gibson, Q. H., Olson, J. S., McKinnie, R. E., & Rohlfs, R. J. (1986) *J. Biol. Chem. 261*, 10228-10239.

Hanson, J. C., & Schoenborn, B. P. (1981) *J. Mol. Biol. 153*, 117-146.

Jajczay, F. L. (1970) Ph.D. Thesis, University of Alberta.

Jongeward, K. A., Magde, D., Taube, D. J., Marsters, J. C., Traylar, T. G., & Sharma, V. S. (1988) *J. Am. Chem. Soc. 110*, 380-387.

Kanaya, E., & Yanagawa, H. (1986) *Biochemistry 25*, 7423-7430.

Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133-154.

Maxwell, J. C., & Caughey, W. S. (1976) *Biochemistry 15*, 388-396.

Mims, M. P., Porras, A. G., Olston, J. S., Noble, R. W., & Peterson, J. A. (1983) *J. Biol. Chem. 258*, 14219-14232.

Morishima, I., Shiro, Y., & Wakino, T. (1985) *J. Am. Chem. Soc. 107*, 1063-1064.

Norvell, J., Nunes, A. C., & Schoenborn, B. P. (1975) *Science (Washington, D.C.) 190*, 568-570.

Ollis, D. L., Appleby, C. A., Colman, P. M., Cutten, A. E., Guss, J. M., Venkatappa, M. P., & Freeman, H. C. (1983) *Aust. J. Chem. 36*, 451-468.

Parkhurst, L. J., Sima, P., & Goss, D. J. (1980) *Biochemistry 19*, 2688-2692.

Phillips, S. E. V. (1980) *J. Mol. Biol. 142*, 531-554.

Phillips, S. E. V., & Schoenborn, B. (1981) *Nature (London) 292*, 81-82.

Rudolph, S. A., Boyle, S. O., Dresden, C. F., & Gill, S. J. (1972) *Biochemistry 11*, 1098-1101.

Shiro, Y., & Morishima, I. (1984) *Biochemistry 23*, 4879-4884.

Stetzkowski, F., Banerjee, R., Marden, M. C., Beece, D. K., Bowne, S. F., Doster, W., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., & Jung, C. (1985) *J. Biol. Chem. 260*, 8803-8809.

Szabo, A., & Perutz, M. F. (1976) *Biochemistry 15*, 4427-4428.

Tucker, P. W., Phillips, S. E. V., Perutz, M. F., Houchens, R., & Caughey, W. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1076-1080.

Wittenberg, J. B., Appleby, C. A., & Wittenberg, B. A. (1972) *J. Biol. Chem. 247*, 527-531.

Crystal Structure of the Carbon Monoxide–Substrate–Cytochrome P-450$_{\mathrm{CAM}}$ Ternary Complex[†,‡]

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ABSTRACT: The crystal structure of the ternary complex formed between carbon monoxide (CO), camphor, and ferrous cytochrome P-450$_{\mathrm{CAM}}$ has been refined to an R value of 17.9% at 1.9-Å resolution. To accommodate the CO molecule, the substrate, camphor, moves about 0.8 Å while at the same time remaining in nonbonded contact with CO. The average temperature factor of the camphor atoms is about 50% higher in the CO complex, suggesting that the camphor is more loosely bound in this ternary complex. The Fe–C–O angle is about 166°, and thus, CO appears to be bent from the heme normal, as it is in various CO–globin complexes, due to steric interactions with active site groups. The oxygen atom of the CO molecule is nestled into a groove formed by an unusual helical hydrogen bond in the distal helix between the highly conserved Thr 252 and Gly 248 residues. In the transition from the ferric camphor-bound binary complex to the ferrous CO–camphor-bound ternary complex, the heme iron atom moves into the plane defined by the pyrrole nitrogens by about 0.41 Å. Although the axial Cys ligand also moves toward the heme, the S–Fe bond stretches from about 2.20 Å in the absence of CO to about 2.41 Å once CO has bound.

Cytochromes P-450 are a group of *b*-type heme proteins that catalyze the hydroxylation of aromatic and aliphatic substrates in a variety of metabolic processes. The most extensively studied P-450 is the camphor hydroxylase from *Pseudomonas putida*, or P-450$_{\mathrm{CAM}}$ (Wagner & Gunsalus, 1982; Gunsalus et al., 1974; Debrunner et al., 1978; Gunsalus & Sligar, 1978). P-450$_{\mathrm{CAM}}$ is a 45 000-dalton polypeptide containing a single ferric protoporphyrin IX. This enzyme converts camphor to 5-*exo*-hydroxycamphor as the first step in the oxidative assimilation of camphor as a carbon source.

The most characteristic feature of all P-450s is the unusual 450-nm Soret band of the ferrous CO complex which is considerably red shifted relative to that of most other ferrous CO

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[‡] The atomic coordinates of the CO–camphor–P-450$_{\mathrm{CAM}}$ structure have been deposited in the Brookhaven Protein Data Bank.

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