Preparation and Reactions of Myoglobin Mutants Bearing both Proximal Cysteine Ligand and Hydrophobic Distal Cavity: Protein Models for the Active Site of P-450†

Toshitaka Matsui, Shingo Nagano,‡ Koichiro Ishimori, Yoshihito Watanabe,*,§ and Isao Morishima*

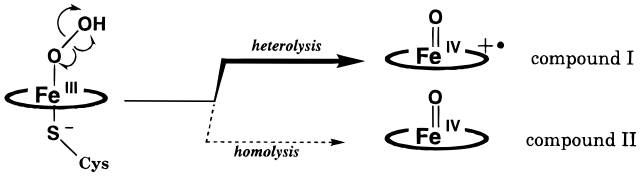
Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 606-01, Japan

Received February 26, 1996; Revised Manuscript Received June 24, 1996⊗

ABSTRACT: We have reported that H93C human myoglobin (Mb), in which proximal histidine (His93, F8) was replaced by cysteine, gave nearly identical spectroscopic features of P-450 [Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., Morishima, I., Egawa, T., Kitagawa, T., & Makino R. (1993) *Biochemistry 32,* 241−252]. More importantly, the thiolate ligand enhanced its oxygenation activities when supported by H_2O_2 due to the exclusive encouragement of heterolytic O−O bond cleavage of peroxides. While we have attributed the enhanced heterolysis to the electron donation from the thiolate ligand, possible participation of the distal histidine (H64, E7) in H93C Mb cannot be eliminated. In addition, the racemic product formation catalyzed by H93C Mb implied that its distal cavity could prevent substrates from accessing to the heme and the reactions may proceed other than by the P-450 type mechanism (ferryl oxygen transfer). In order to clarify whether the distal histidine is involved in the O−O bond cleavage step and to improve accessibility of substrates, the distal histidine of H93C Mb is replaced by smaller and nonpolar residues, glycine (H64G/H93C Mb) and valine (H64V/H93C Mb), by site-directed mutagenesis. Various spectroscopic studies on these double-mutated Mbs revealed the ligation of cysteine to the ferric heme as a thiolate form. In the reaction with cumene hydroperoxide, the anionic nature of the proximal cysteine in H64G/H93C and H64V/H93C Mbs was found to encourage the heterolytic O−O bond cleavage as observed for H93C Mb. The results clearly demonstrate that the distal histidine of H93C Mb is hardly involved in the O−O bond cleavage step and are in good agreement with the role of thiolate ligation for the formation of the reactive intermediate, equivalent to compound **I**, in the catalytic cycle of P-450 reactions. In the oxygenation of methyl *p*-tolyl sulfide, the ratios of ferryl oxygen transfer increased in H64G/H93C Mb (58%) and H64V/H93C Mb (78%) as compared to H93C Mb (53%). The increased ratios of ferryl oxygen transfer imply the active site of H64G/H93C and H64V/H93C Mbs being more accessible for substrates; however, the sulfoxidation by the ferric mutant Mbs/H_2O_2 system was much slower than that by H93C Mb. The poor activities of these mutant Mbs are attributed to the significantly discouraged binding of H_2O_2.

P-450 is a heme-containing monooxygenase, which activates molecular oxygen by utilizing two electrons and inserts one oxygen (oxygenation) to various organic substrates. Biological activities and unique spectroscopic features of P-450 are attributed to unusual cysteine coordination to heme iron as an anionic thiolate ligand (Ortiz de Montellano, 1986; Dawson & Sono, 1987; Dawson, 1988). The active species responsible for those oxygenations is assumed to be a high-valent oxo species produced via heterolytic O−O bond cleavage of a putative hydroperoxoiron(III) complex (Scheme 1). Strong electron donation from the thiolate ligand is expected to destabilize the O−O bond and facilitates its heterolytic cleavage to afford an oxoiron(IV) porphyrin π-cation radical (O═Fe^{IV}−$Por^{+\bullet}$), so-called compound **I** (Peisach, 1975; Dawson et al., 1976; Dawson & Sono, 1987; Dawson, 1988). When such strong donation is absent, the ratio of the homolytic cleavage over heterolysis can be increased to give a less reactive O═Fe^{IV}−Por intermediate (compound **II**).

Scheme 1



In order to examine these hypothetical roles of the thiolate ligation, Adachi et al. (1991, 1993) replaced the proximal histidine (His93, F8) of human myoglobin (Mb)[1] by cysteine (H93C Mb). The cysteine ligand of H93C Mb was found to exclusively accelerate the heterolysis of the O−O bond in the reaction with cumene hydroperoxide. Furthermore, H93C Mb exhibited nearly identical spectroscopic properties with those of P-450. While the replacement of the proximal ligand with cysteine allowed us to alter some properties of

† This work is supported by a grant from the Ministry of Education, Science and Culture (07309006 to I.M.).

* To whom correspondence should be addressed. E-mail: morisima@mds.moleng.kyoto-u.ac.jp. Phone: +81-75-753-5921. Fax: +81-75-751-7611.

‡ Present address: Department of Biochemistry, School of Medicine, Keio University, Shinanomachi 35, Shinjyuku-ku, Tokyo, 160, Japan.

§ Present address: Institute for Molecular Science, Okazaki 444, Japan.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: Mb(s), myoglobins(s); P-450cam(+cam), *d*-camphor-bound P-450cam.

S0006-2960(96)00459-X CCC: $12.00

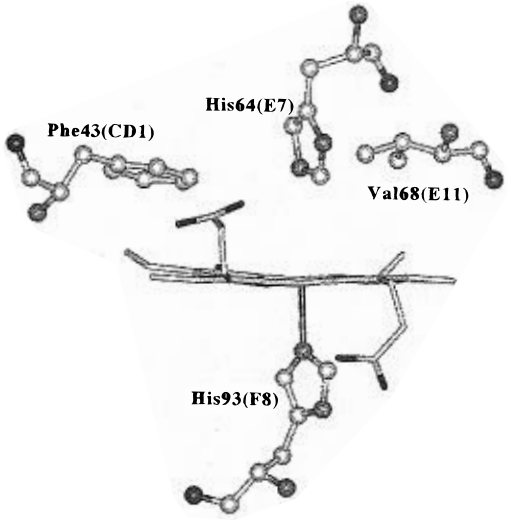


FIGURE 1: Heme environmental structure of myoglobin. Heme and some selected residues including proximal His93(F8) and distal His64(E7) are shown.

Mb to those of P-450, there are still major differences between H93C Mb and P-450. For example, distal residues of P-450s are usually nonpolar, and it is hard to find a possible general acid−base catalyst such as histidine in peroxidases (Poulos et al., 1980). Apparently, there is a histidine residue (His64) in the distal site of H93C Mb, which is capable of serving as the general acid−base catalyst even though no participation of the distal histidine of wild-type Mb in the O−O bond cleavage has been reported (Allentoff et al., 1992).

More importantly, racemic epoxide of styrene by a H93C Mb/H_2O_2 system implied that the oxygenations were not carried out in its distal cavity. Since similar racemic epoxidation by wild-type Mb/H_2O_2 has been attributed to the oxidation with the activated molecular oxygen by a surface tyrosine radical (Ortiz de Montellano & Catalano, 1985; Choe et al., 1994), H93C Mb would catalyze epoxidation by the same surface radical mechanism rather than the P-450-type, direct ferryl oxygen transfer mechanism. These mechanistic differences between Mbs and P-450 would be derived from the hindered distal cavities of both wild-type and H93C Mbs to retain organic substrates (Figure 1). In fact, replacement of the distal histidine of Mb by a smaller residue resulted in a higher ratio of P-450-type epoxidation than wild-type Mb (Rao et al., 1993).

In this paper, we report the further mutations on the distal histidine of H93C human Mb to smaller and nonpolar residues, glycine (H64G/H93C Mb) and valine (H64V/H93C Mb). The elimination of the distal histidine will allow examination of a possible involvement of the residue in the heterolytic and homolytic O−O bond cleavages and will give more precise information on roles of the thiolate ligand in the P-450-catalyzed reactions. At the same time, the enlarged or more hydrophobic distal cavity could change the oxygenation to the P-450-type process. Coordination structures of these two double-mutated Mbs, especially ligation of cysteine, have been studied by various spectroscopic methods. The oxygenation activities and reaction mechanisms have been examined by peroxide-supported sulfoxidation of methyl *p*-tolyl sulfide and compared with those of wild-type and H93C Mbs.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Protein Preparation, and Purification. The original expression vector of human Mb gene including the lead protein, pMb3 (pLcIIFXMb), is a gift from Varadarajan and Boxer (Varadarajan et al., 1985). The procedures for site-directed mutagenesis and DNA sequencing were described elsewhere (Adachi et al., 1991). Preparation and purification of wild-type and mutant Mbs were performed according to a method described by Varadarajan et al. (1989). However, digestion of the leader peptide (31 amino acids from the cII protein) was only conducted to wild-type and H93C Mbs. H64G/H93C and H64V/H93C Mbs could be purified to give a single band as monitored by SDS−PAGE. Nevertheless, the double mutants were unstable against the tryptic digestion, though the corresponding single mutants such as H93C and H64G Mbs are stable for digestion (Adachi et al., 1991; Nagano, unpublished data).

It has been shown that the electronic and environmental structure of heme is nearly identical for the fusion and digested wild-type Mb (Shiro et al., 1994). Similarly, ferric fusion H93C Mb gave ^{1}H-NMR and resonance Raman spectra nearly identical with those of the digested one (data not shown). We also confirmed that the leader peptide of H93C Mb had no considerable effect on its N-demethylation activity of *N*,*N*-dimethylaniline and cleavage mode of the peroxide O−O bond. Therefore, it seemed unlikely that the additional sequence of H64G/H93C and H64V/H93C Mbs altered their essential features, and we used the double mutants without a trypsin treatment for further studies.

Spectroscopy. All spectroscopic measurements were performed in 50 mM sodium phosphate buffer (pH 7.0) unless stated. Electronic absorption spectra were recorded on a Shimadzu UV-2200 spectrometer, and the concentration of the samples was 10 μM except for the deoxy band III (ca. 200 μM). Ferrous Mbs were prepared by addition of a small excess amount of sodium dithionite, and the following CO gas bubbling afforded ferrous carbonmonoxy Mbs. Extinction coefficients were determined by the pyridine−hemochrome method (Antonini & Brunori, 1971).

Hyperfine-shifted ^{1}H-NMR spectra in 0.1 M sodium phosphate (pD 7.0) were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system (Ishimori & Morishima, 1986, 1988). Chemical shifts are referenced to HDO, and the concentration of the samples was ca. 1 mM.

Electronic paramagnetic resonance (EPR) measurements were carried out at 5.0 K and at X-band (9.35 GHz) microwave frequency with a JEOL JES-3X spectrometer operating with 100 kHz magnetic field modulation. The concentration of the samples was ca. 0.5 mM.

Reaction with Cumene Hydroperoxide. All the reactions were performed in 50 mM sodium phosphate (pH 7.0), and organic compounds were added as a methanol solution (Adachi et al., 1993). H_2O_2 was standardized with potassium permanganate.

A 1 mL solution of ferric wild-type or mutant Mb (10 μM) and *N*,*N*-dimethylaniline (1 mM) was preincubated at 20 °C for 20 min. Incubation was initiated by addition of cumene hydroperoxide (final concentration 30 μM) to the preincubated solution. During the incubation, aliquots of the solution (10 μL) were directly loaded on a high-pressure

liquid chromatograph (Waters 600 equipped with Waters 741 data module). A reverse-phase HPLC column (Waters μBondasphere 5 μm CN-100 Å) was employed to determine the reaction products (internal standard: benzyl alcohol), which were eluted at a flow rate of 0.7 mL/min with water containing 10% methanol. The eluent was monitored at 210 nm with a Waters Lambda-Max Model 481 LC spectrophotometer, and assignment of the components was based on the retention time of authentic samples. The reaction and the product analysis was duplicated for each set of experimental points.

Measurement of Oxidation Activities. Sulfoxidation of methyl *p*-tolyl sulfide was performed as described in the preceding paragraph. The concentration of methyl *p*-tolyl sulfide and H_2O_2 was 0.5 and 0.2 mM, respectively. The products were eluted at a flow rate of 0.6 mL/min with water containing 30% methanol (internal standard: propiophenone). Assignment of the components was based on the retention time of authentic samples and GLC−MASS analysis (Shimadzu GC-14A gas chromatograph coupled to a Shimadzu QP200S mass spectrometer).

N-Demethylation activities of *N*,*N*-dimethylaniline by the mutants were determined by the amount of formaldehyde formed. Incubation and workup were carried out as described by Adachi et al. (1993).

Iodide oxidation activities were measured at 20 °C by following formation of I_3^- ($\epsilon_{353} = 26.2$ mM^{-1} cm^{-1}; Ramette & Sanford, 1965). The concentration of Mb, H_2O_2, and potassium iodide was 1 μM, 1 mM, and 20 mM, respectively.

Oxygen Source for Sulfoxidation of Methyl p-Tolyl Sulfide. $H_2{}^{18}O_2$ was prepared from $^{18}O_2$ as described by Sawaki and Foote (1979). The ^{18}O content of the peroxide was determined to be 92% by epoxidation of menadione (Ortiz de Montellano & Catalano, 1985). A 5 mL reaction mixture containing either ferric wild-type or mutant Mb (40 μM) and methyl *p*-tolyl sulfide (5 mM, not completely dissolved) was preincubated for 20 min at 25 °C. $H_2{}^{18}O_2$ was added to initiate the reaction (final concentration 2.0 mM), and the mixture was incubated under aerobic conditions at 25 °C for 1 h. The incubation mixture was then extracted with diethyl ether at least three times (total 10 mL) by using centrifugation. The extract was dried over anhydrous sodium sulfate, and most of diethyl ether was evaporated. Then, metabolites were analyzed by GLC−MASS to determine the ratio of the oxygen derived from $H_2{}^{18}O_2$.

RESULTS

Electronic Absorption Spectra. Table 1 summarizes the absorption maxima and extinction coefficients of H64G/H93C and H64V/H93C human Mbs. The Soret bands of the ferric (Fe^{3+}) double-mutated Mbs are less intense and blue shifted as compared to that of wild-type Mb. These spectral features including the Q-band absorption resemble those of P-450 and H93C Mb, particularly the Soret maxima at 391 nm (Yu et al., 1974; Adachi et al., 1991). Other five-coordinate ferric high-spin hemoproteins also show similar spectra; however, the Soret maxima are observed at somewhat longer wavelength (Mintorovitch & Satterlee, 1988; Ikeda-Saito et al., 1992; Yu et al., 1974; Adachi et al., 1991). In addition, the spectra of ferric H64G/H93C and H64V/H93C Mbs are analogous to that of a similar mutant (H64V/H93C) of horse heart Mb in which proximal cysteine has been shown to serve as an axial ligand (Hildebrand et al., 1995). Thus, ferric H64G/H93C and H64V/H93C human Mbs are suggested to be in thiolate-bound five-coordinate states similar to those of ferric P-450cam(+cam) and H93C Mb (Poulos et al., 1985; Adachi et al., 1993).

Table 1: Wavelength (Extinction Coefficients) of Absorption Maxima for Some Forms of Wild-Type and Mutant Myoglobins

protein		Soret[a]	visible (band III)	
H64G/H93C	Fe^{3+}	391 (95)	508	637
	Fe^{2+}	428	568	(770)
	$Fe^{2+}CO$	421	541	569
H64V/H93C	Fe^{3+}	391 (100)	508	637
	Fe^{2+}	428	570	(769)
	$Fe^{2+}CO$	421	540	569
H93C[b]	Fe^{3+}	391 (115)	509	629
	Fe^{2+}	428	558	(763)
	$Fe^{2+}CO$	420	539	569
wild type	Fe^{3+}	408 (184)	502	632
	Fe^{2+}	433	558	(761)
	$Fe^{2+}CO$	422	541	579
P-450cam(+cam)[c]	Fe^{3+}	391 (91)	540	646
	Fe^{2+}	408	540	(−)
	$Fe^{2+}CO$	447	550	

[a] Extinction coefficients are given in parentheses (cm^{-1} mM^{-1}). [b] Adachi et al. (1991). [c] Yu et al. (1974).

In the ferrous deoxy (Fe^{2+}) and carbonmonoxy ($Fe^{2+}CO$) forms, H64G/H93C and H64V/H93C Mbs showed quite different spectra from those of P-450 (Table 1). Especially, the characteristic Soret absorption around 450 nm of carbonmonoxy P-450 could not be reproduced by the double-mutated Mbs. These results imply that H64G/H93C and H64V/H93C Mbs lose the thiolate ligands upon reduction as well as H93C Mb and the horse heart mutant (Adachi et al., 1993; Hildebrand et al., 1995). Though ferrous H93C Mb was shown to be coordinated by the distal histidine, such a ligation structure is ruled out in the double-mutated Mbs because the residue has been replaced.

The failure of the thiolate coordination in ferrous states was also suggested by the position of band III absorption in deoxy Mbs (Table 1), which is known to shift to longer wavelength when heme iron is closer to the porphyrin plane (Jackson et al., 1994). Because of either proximal or distal histidine pulling heme iron, the band III absorption of deoxy wild-type and H93C Mbs appeared at 761 and 763 nm, respectively. On the other hand, the band III of H64G/H93C and H64V/H93C Mbs was red shifted toward 770 and 769 nm, respectively. These results demonstrate that the heme irons of deoxy H64G/H93C and H64V/H93C Mbs are closer to the heme plane, which is consistent with the loss of strong axial ligands.

Proton Nuclear Magnetic Resonance in the Ferric States. Figure 2 presents hyperfine-shifted ^{1}H-NMR spectra of ferric human Mbs and P-450cam(+cam) at room temperature. The intense peaks in these spectra are assigned to the heme methyl groups. The signals for wild-type Mb were observed at 86.5, 80.3, 67.4, and 48.1 ppm, far downfield from the HDO resonance (Morishima et al., 1978), whereas the corresponding signals of the mutants are located at 57.8, 49.8, 36.8, and 27.3 ppm for H64G/H93C Mb and at 58.9, 49.5, 36.8, and 27.9 ppm for H64V/H93C Mb. The resonance positions of the heme methyl groups in these two mutant Mbs are nearly identical with those of H93C Mb, P-450cam(+cam), and the H64V/H93C mutant of horse heart Mb

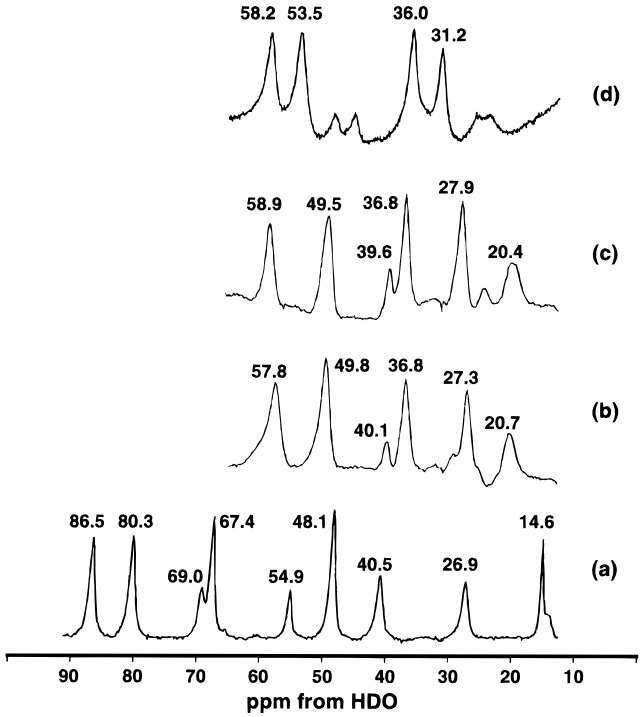


FIGURE 2: ^{1}H-NMR spectra of ferric high-spin forms of (a) human wild-type, (b) H64G/H93C, and (c) H64V/H93C Mbs and (d) P-450cam(+cam) in 100 mM sodium phosphate buffer at pD 7.0 and at 23 °C. Concentration of samples is ca. 1 mM.

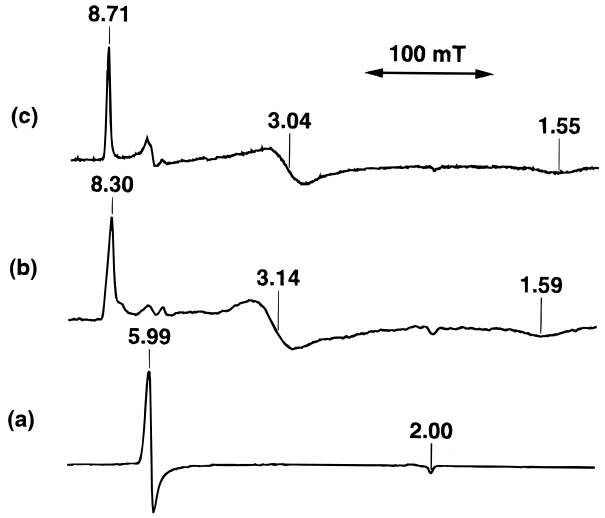


FIGURE 3: EPR spectra of ferric high-spin forms of (a) human wild-type, (b) H64G/H93C, and (c) H64V/H93C Mbs in 50 mM sodium phosphate buffer at pH 7.0 and at 5.0 K. Concentration of samples is ca. 0.5 mM. The *g* values are indicated on the figure.

(Adachi et al., 1991: Hildebrand et al., 1995). These results indicate that ferric hemes of H64G/H93C and H64V/H93C Mbs exhibit electronic structures similar to those of H93C Mb and P-450cam(+cam), presumably due to the thiolate coordination.

Electron Paramagnetic Resonance Spectra in the Ferric States. The thiolate ligation in ferric H64G/H93C and H64V/H93C Mbs is also confirmed by EPR spectroscopy at 5 K (Figure 3). While wild-type Mb gave typical high-spin signals with the axial ligand field symmetry, the mutant Mbs exhibited very rhombic high-spin signals. These high rhombicities are characteristic of ferric high-spin hemoproteins bearing thiolate ligand, and the observed *g* values are

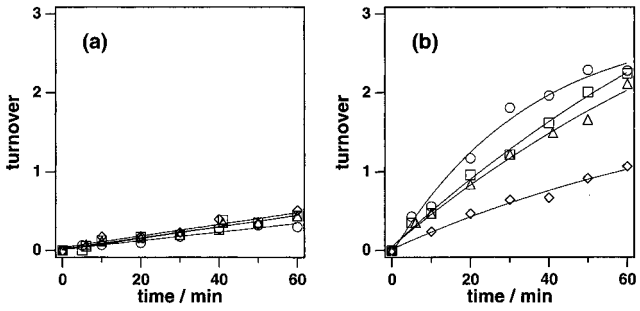


FIGURE 4: Time course of (a) acetophenone and (b) cumyl alcohol formation in the reaction of 10 μM Mbs (◇, wild-type; □, H64G/H93C; △, H64V/H93C; ○, H93C Mbs) with 30 μM cumene hydroperoxide. Experiments were performed in 50 mM sodium phosphate buffer at pH 7.0 and at 20 °C in the presence of *N*,*N*-dimethylaniline as a substrate.

similar to those reported for H93C Mb, P-450, and chloroperoxidase (Adachi et al., 1993; Tsai et al., 1970; Peisach & Blumberg, 1970; Hollenberg et al., 1980).

H64G/H93C and H64V/H93C Mbs exhibited no evidence for the low-spin species, though low-spin signals were found in the spectra of P-450 and H93C Mb (Adachi et al., 1993; Tsai et al., 1970; Peisach & Blumberg, 1970). Adachi et al. (1993) suggested that a sixth ligand, probably water, coordinated in H93C Mb to give low-spin species at low temperature. Since it is well-known that distal histidine of ferric Mb stabilizes a coordinated water molecule (Antonini & Brunori, 1971), the replacement of distal histidine in H64G/H93C and H64V/H93C Mbs is likely to destabilize the sixth ligand. The H64V/H93C mutant of horse heart Mb also lacked low-spin signals (Hildebrand et al., 1995), and they also noted a similar interpretation. Apparently, these differences in the ligation state occur only at low temperature because absorption and NMR spectra assure all the cysteine mutant Mbs being five-coordinate high-spin forms at room temperature.

Reactions of Ferric Wild-Type and Mutant Mbs with Cumene Hydroperoxide. In reactions with various oxidants, any attempts to detect higher valent species of H64G/H93C and H64V/H93C Mbs failed. Thus, reactions of ferric wild-type and mutant Mbs with cumene hydroperoxide have been carried out in order to examine effects of the proximal and distal histidine substitutions on the formation of active species. It is known that heterolytic O−O bond cleavage of cumene hydroperoxide affords a compound **I** equivalent and cumyl alcohol (eq 1). On the other hand, homolysis of the O−O bond gives less reactive compound **II** and a cumyloxy radical which subsequently eliminates a methyl radical to afford acetophenone (eq 2 and 3). To avoid possible self-destruction of the heme by high-valent intermediates, *N*,*N*-dimethylaniline was added as a substrate. Time courses of product formation are shown in Figure 4 (see Table 2 for product analysis).

$$Fe^{III}Por + PhC(CH_3)_2OOH \xrightarrow{\text{heterolysis}} O{=}Fe^{IV}Por^{+\bullet} + PhC(CH_3)_2OH \quad (1)$$

$$Fe^{III}Por + PhC(CH_3)_2OOH \xrightarrow{\text{homolysis}} O{=}Fe^{IV}Por + PhC(CH_3)_2O^{\bullet} + H^{+} \quad (2)$$

$$PhC(CH_3)_2O^{\bullet} \rightarrow PhCOCH_3 + CH_3^{\bullet} \quad (3)$$

Table 2: Product Analysis in the Reactions of Ferric Hemoproteins with Cumene Hydroperoxide

protein	$PhC(CH_3)_2OH$[a]	$PhCOCH_3$[a]	hetero/homo[b]
H64G/H93C	4.8	0.71	87/13
H64V/H93C	4.2	0.70	86/14
H93C	5.6	0.65	89/11
wild type	1.8	0.80	69/31
P450cam[c]			86/14

[a] 10^{-2} nmol of product/nmol of Mb•min^{-1}. [b] Ratio of $PhC(CH_3)_2OH$/$PhCOCH_3$. [c] Nagano, unpublished result.

Table 3: Initial Reaction Rates Catalyzed by Wild-Type and Mutant Myoglobins

	sulfoxidation of methyl *p*-tolyl sulfide[a]		N-demethylation of *N*,*N*-dimethylaniline[b]
myoglobin	H_2O_2	CHPO[c]	H_2O_2
H64G/H93C	4.7	9.5	nm[d]
H64V/H93C	4.1	5.5	nm[d]
H93C	83	19	213
wild type	5.5	3.8	107

[a] 10^{-2} nmol of sulfoxide/nmol of Mb•min^{-1}. [b] nmol of HCHO/nmol of Mb•min^{-1}. [c] Cumene hydroperoxide. [d] Not measurable.

Under the conditions we employed, wild-type and the mutant Mbs exhibited nearly identical rates for the formation of $PhCOCH_3$, which corresponds to the homolysis (Figure 4a). On the other hand, the formation of the heterolysis products, $PhC(CH_3)_2OH$, was obviously accelerated in the three cysteine-substituted Mbs as compared to wild-type Mb (Figure 4b). Although these formation rates can be influenced by factors other than the cleavage rates (see below), the heterolysis/homolysis ratios are the direct indication of the thiolate ligation effect on the formation of the compound **I** equivalent (Table 2).

Table 2 clearly shows that H64G/H93C and H64V/H93C Mbs exhibit greater heterolysis ratios than wild-type Mb, and the ratios of the double mutants are almost the same as that of P-450cam. H93C Mb afforded a little higher heterolysis ratio than H64G/H93C and H64V/H93C Mbs; however, the difference among the mutant Mbs seems to be small. These observations are consistent with the preserved heterolysis/homolysis ratio through the replacement of the distal histidine in wild-type Mb (Allentoff et al., 1992). Since these results reveal that H64G/H93C and H64V/H93C Mbs afford a highly reactive species in the reaction with peroxides as observed for H93C Mb, the participation of the distal histidine in the mutant Mbs is apparently less effective than the thiolate ligation for the increased heterolysis. Therefore, we can conclude that the anionic thiolate ligand, even without any assistance by the distal histidine, can facilitate the formation of highly reactive species by selective enhancement of the heterolytic O−O bond cleavage.

Oxidation Activities of Wild-Type and Mutant Mbs. Since the thiolate ligation in H64G/H93C and H64V/H93C Mbs was lost upon reduction, oxygenation activities were measured by utilizing a peroxide shunt path in the P-450-catalyzed reactions. Initial reaction rates for H_2O_2-dependent sulfoxidation of methyl *p*-tolyl sulfide and N-demethylation of *N*,*N*-dimethylaniline are listed in Table 3. The metabolites of methyl *p*-tolyl sulfide by ferric Mb and H_2O_2 were identified by GLC−MASS analysis as the corresponding sulfoxide and sulfone. Because of the amount of sulfone being negligible in comparison with sulfoxide, we determined the reaction rates only for the sulfoxide formation. Initial sulfoxidation rate of H93C Mb supported by H_2O_2 was 15 times greater than that of wild-type Mb. However, H64G/H93C and H64V/H93C Mbs showed nearly identical activities with wild-type Mb even though the mutant Mbs were shown to facilitate heterolytic peroxide bond cleavage. In N-demethylation of *N*,*N*-dimethylaniline, activities of H64G/H93C and H64V/H93C Mbs were too small to measure.

Table 4: Iodide Oxidation Activities and Oxygen Source in the Sulfoxidation of Methyl *p*-Tolyl Sulfide

myoglobin	iodide oxidation[a]	oxygen derived from H_2O_2/%
H64G/H93C	1.6	58
H64V/H93C	0.58	78
H93C	42	53
wild type	1.4	98

[a] nmol of I_3^-/nmol of Mb•min^{-1}.

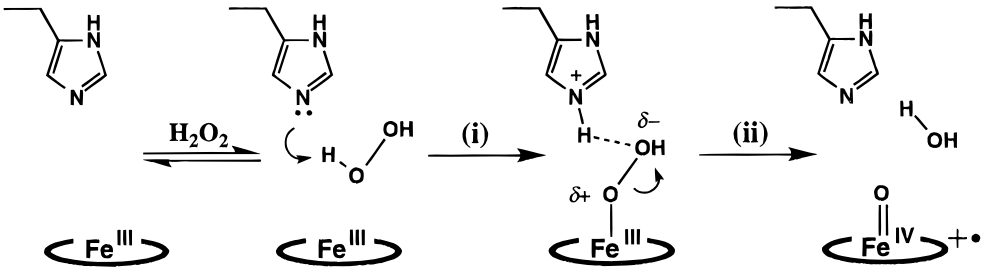
Cumene hydroperoxide was also employed as an oxidant instead of H_2O_2 (Table 3). Interestingly, sulfoxidation activities of wild-type and H93C Mbs were reduced in comparison with those supported by H_2O_2 while the activities of H64G/H93C and H64V/H93C Mbs increased. Upon the N-demethylation reactions, similar peroxide dependency of the activities has been observed for the wild-type and mutant Mbs (data not shown). These observations indicate that the reactions of peroxide with Mbs are involved in the rate-determining step of the oxidations of the substrates by the Mbs.

We have also examined the H_2O_2-supported oxidation of iodide to I_3^- (Table 4). Because of the great reactivity of iodide, the oxidation rates of iodide could be a good measure of the active species formation. Thus, the extremely high iodide oxidation activity of H93C Mb is attributed to its high reactivity with H_2O_2, and the low activities of the double-mutated Mbs suggest their decreased reactivities with H_2O_2, especially in the H64V/H93C mutant. Considering the facile heterolysis by the axial thiolate (Table 2), the poor reactivities with H_2O_2 could be ascribed to its slow binding to heme iron by the replacement of distal histidine.

Origin of the Oxygen in Methyl p-Tolyl Sulfoxide. In order to evaluate whether the reactions proceed through P-450-type ferryl oxygen transfer or other mechanisms, $H_2{}^{18}O_2$-supported sulfoxidation of methyl *p*-tolyl sulfide has been examined under aerobic conditions. Table 4 summarizes the ratios of the incorporated oxygen derived from H_2O_2. In the peroxide-dependent oxygenations by P-450, peroxide oxygen is incorporated into the metabolites (Nordblom et al., 1976). As previously suggested by Adachi et al. (1993), the sulfoxide oxygen from H_2O_2 in the H93C Mb catalyzed sulfoxidation was only 53%. The increased ratios for H64G/H93C and H64V/H93C Mbs (58% and 78%, respectively) indicate the alteration of the oxygenation mechanism rather close to that of P-450 by engineering of the distal cavity. However, the small improvement observed in H64G/H93C Mb in spite of its largest distal cavity suggests that the size of the distal cavity is not the sole factor for the efficient P-450-type oxygenations. The hydrophobicity of the active site rather seems to be crucial for the sulfoxidation by the cysteine-substituted Mbs.

In contrast to the mutant Mbs, wild-type Mb exclusively catalyzed the sulfoxidation by ferryl oxygen transfer (98%).

Scheme 2



Since the structures of the distal cavity are expected to be very similar between wild-type and H93C Mbs, the decreased ratio of P-450-type oxygenations could be attributed to rapid diffusion of the oxidizing equivalent in the mutant Mbs rather than poor accessibility of sulfides.

DISCUSSION

Coordination Structures of Mutant Mbs. Various spectroscopic features of H64G/H93C and H64V/H93C Mbs are essentially the same as those of H93C Mb and the H64V/H93C mutant of horse heart Mb (Adachi et al., 1993; Hildebrand et al., 1995). In the ferric forms, these mutant Mbs gave spectra similar to those for P-450 and porphyrin−thiolate complexes (Collman et al., 1975; Koch et al., 1975). Nevertheless, the absorption spectra of the ferrous Mbs are clearly distinct from those of P-450 (Table 1). These results show that the thiolate ligands in ferric H64G/H93C and H64V/H93C Mbs are eliminated upon reduction as observed for the other cysteine mutant Mbs (Adachi et al., 1993; Hildebrand et al., 1995). The size of the cysteine residue seems too short to form a stable ligation state in place of the proximal histidine. The successful coordination of the thiolate ligand to the ferric Mbs could be achieved by the interaction of positively charged ferric heme (Fe^{3+}/Por^{2-}) with the anionic thiolate.

The absorption spectra of deoxy H64G/H93C and H64V/H93C Mbs resemble those of hemoproteins which lack internal axial ligands (Palaniappan & Bocian, 1994; Sun et al., 1994; McRee et al., 1994). Especially, nearly identical spectra with acid-denatured deoxy Mb (Soret ∼426 nm, band III ∼773 nm) are indicative of these two mutants being in aquo five-coordinate states as suggested for the denatured Mb (Palaniappan & Bocian, 1994). Detailed structures of the CO forms remain unclear concerning an axial ligand and CO binding side. Since five-coordinated model CO complexes exhibit Soret maxima at ∼390 nm (Traylor et al., 1985), the CO forms of the double mutants seem to be six-coordinated as suggested for the H64V/H93C mutant of horse heart Mb (Hildebrand et al., 1995). While a reasonable candidate for the sixth ligand would be water, we cannot rule out other possibilities.

Roles of the Distal Histidine of Mutant Mbs in the Formation of Active Species. In the catalytic cycle of peroxidase reactions, the distal histidine of peroxidases is considered to serve as a general acid−base catalyst as shown in Scheme 2. In the first step, the distal histidine reacts with H_2O_2 as the general base to abstract a proton and affords a putative hydroperoxoiron(III) complex. A protonated form of the histidine then serves as the general acid to facilitate heterolytic O−O bond cleavage (Poulos & Kraut, 1980). In fact, the replacements of the distal histidine in peroxidases drastically decrease the rate of compound **I** formation (Erman et al., 1993; Newmyer et al., 1995).

However, as we have shown in Table 2, the replacement of the distal histidine in H93C Mb to valine and glycine did not cause very much change in the heterolysis/homolysis ratio of the O−O bond of cumene hydroperoxide. Very similar observations were reported upon the elimination of the distal histidine in sperm whale Mb (Allentoff et al., 1992). Thus, the distal histidine in Mb can be less important in the O−O bond cleavage step. At the same time, we have observed quite lower oxidation activity of H64G/H93C and H64V/H93C Mbs than H93C Mb when H_2O_2 was employed as an oxidant. Thus, the distal histidine in H93C Mb is a crucial amino acid residue to utilize H_2O_2 as the oxidant while it does not play an important role on the O−O bond cleavage step. These results suggest involvement of the distal histidine in the binding of H_2O_2 to the heme. There are a couple of possible roles of the distal histidine in H93C Mb: (i) it serves as a general base to encourage the formation of the hydroperoxo-iron complex, (ii) replacement of the distal histidine to valine causes the active site environment to be less polar to prevent the incorporation of H_2O_2 into the active site, and (iii) the presence of the distal histidine makes the active site less spacious while glycine could afford a less hindered active site. The role of the distal histidine in Mb as the general base has been pointed out in the binding of cyanide to Mb (Brancaccio et al., 1994). The association rate constants of CN^- to Mbs were significantly decreased by the replacement of the distal histidine: WT, 2.3×10^{-1}; H64A, 1.1×10^{-1}; H64V, 2.8×10^{-2}; H64L, 3.4×10^{-3} mM^{-1} s^{-1} (Brancaccio et al., 1994). Since the cyanide binding at neutral pH requires deprotonation from its protonated form (pK_a of cyanide: ∼9), its facile binding to the wild-type Mb was attributed to the presence of the distal histidine as a general base catalyst.

Lower cumene hydroperoxide-supported sulfoxidation activity of H93C Mb than H_2O_2-supported activity could be the reflection of the sterically hindered active site structure, even though the distal histidine could serve as the general base. Thus, the differences in relative activities of H64G/H93C and H64V/H93C Mbs over H93C Mb are smaller upon the use of cumene hydroperoxide in comparison with the cases of H_2O_2. The replacement of the distal histidine to less polar and smaller amino acids is expected to form a less polar and less hindered active site. At the same time, the introduction of glycine could allow the introduction of a solvent water molecule as observed for H64G Mb (Quillin et al., 1993). These effects could finally make different reactivities of H64G/H93C and H64V/H93C Mb mutants.

Mechanism of Sulfoxidation Reactions. Ortiz de Montellano and co-workers extensively examined the oxygenation

mechanism of Mbs (Ortiz de Montellano & Catalano, 1985; Rao et al., 1993; Choe et al., 1994; Rao et al., 1994). They showed that Mb hardly catalyzed ferryl oxygen transfer to various substrates in contrast to P-450. In the styrene epoxidation, only 27% of epoxide catalyzed by Mb was produced through the ferryl oxygen transfer mechanism. However, dominant ferryl oxygen transfer was observed in the sulfoxidation of methyl *p*-tolyl sulfide by wild-type Mb (Table 4). Since it was reported that hemoglobin, a closely resembled protein with Mb, catalyzed sulfoxidation of thianthrene 5-oxide through primarily ferryl oxygen transfer (Alvarez & Ortiz de Montellano, 1992), these mechanistic changes are readily attributed to the efficient reactivities of sulfides than olefins. Thus, methyl *p*-tolyl sulfide can be a good substrate to react with active species equivalent to compound **I** before the diffusion of oxidizing equivalents to neighboring amino acids.

Though the ratio of P-450-type sulfoxidation by H93C Mb was only 53%, the absolute rate of the P-450-type sulfoxidation (overall rate of the sulfoxidation × percentage of ferryl oxygen transfer) is much higher than that of wild-type Mb (Tables 3 and 4). Greater sulfoxidation by the H93C Mb indicates that the substrate is able to access the active site of the mutant; thus, we attribute the small incorporation of ^{18}O by H93C Mb to the competent leakage of oxidizing equivalent due to much higher reactivities of the species equivalent of compound **I**; i.e., greater reactivity of the active species of the mutant Mbs could compete the direct oxidation of the substrates with the oxidation of amino acid residues to afford a surface radical as proposed in the epoxidation reactions by Mb (Ortiz de Montellano & Catalano, 1985).

In summary, two double-mutated human myoglobins (H64G/H93C and H64V/H93C Mbs) are prepared by site-directed mutagenesis. Various spectroscopic studies support the cysteine coordination to ferric heme as a thiolate form but not to a ferrous heme. The axial thiolate ligand has been shown to enhance heterolytic O−O bond cleavage of the peroxide even without the assistance of the distal histidine. These observations imply a crucial role of the thiolate ligand in the formation of active species of P-450 in which the distal cavity consists of less polar amino acids. In our laboratory, direct estimation on the axial ligand effect in P-450 is now under investigation using an axial ligand mutant of P-450cam. On the other hand, elimination of the distal histidine decreased the H_2O_2-supported oxidation activities of the double-mutated Mbs probably due to the significantly discouraged H_2O_2 binding to the heme.

ACKNOWLEDGMENT

We are grateful to Dr. Hiroshi Hori (Osaka University) for his kind help in EPR measurement.

REFERENCES

Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., & Morishima, I. (1991) *Biochem. Biophys. Res. Commun. 180*, 138−144.
Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., & Morishima, I., Egawa, T., Kitagawa, T., & Makino R. (1993) *Biochemistry 32*, 241−252.
Allentoff, A. J., Bolton, J. L., Wilks, A., Thompson, J. A., & Ortiz de Montellano, P. R. (1992) *J. Am. Chem. Soc. 114*, 9744−9749.
Alvarez, J. C., & Ortiz de Montellano, P. R. (1992) *Biochemistry 31*, 8315−8322.
Brancaccio, A., Cutruzzola, F., Allocatelli, C. T., Brunori, M., Smerdon, S. J., Wilkinson, A. J., Dou, Y., Keenan, D., Ikeda-Saito, M., Brantley, R. E., Jr., & Olson, J. S. (1994) *J. Biol. Chem. 269*, 13843−13853.
Choe, Y. S., Rao, S. I., & Ortiz de Montellano, P. R. (1994) *Arch. Biochem. Biophys. 314*, 126−131.
Collman, J. P., Sorrell, T. N., & Hoffman, B. M. (1975) *J. Am. Chem. Soc. 97*, 913−914.
Dawson, J. H. (1988) *Science 240*, 433−439.
Dawson, J. H., & Sono, M. (1987) *Chem. Rev. 87*, 1255−1276.
Dawson, J. H., Holm, R. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., & Tang, S. C. (1976) *J. Am. Chem. Soc. 98*, 3707−3709.
Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., & Kraut, J. (1993), *Biochemistry 32*, 9798−9806.
Hildebrand, D. P., Ferrer, J. C., Tang, H.-L., Smith, M., & Mauk, A. G. (1995), *Biochemistry 34*, 11598−11605.
Hollenberg, P. F., Hager, L. P., Blumberg, W. E., & Peisach, J. (1980) *J. Biol. Chem. 255*, 4801−4807.
Ikeda-Saito, M., Hori, H., Andersson, L. A., Prince, R. C., Pickering, I. J., George, G. N., Sanders, C. R., Lutz, R. S., McKelvey, E. J., & Mattera, R. (1992) *J. Biol. Chem. 267*, 22843−22852.
Ishimori, K., & Morishima, I. (1986) *Biochemistry 25*, 4892−4898.
Ishimori, K., & Morishima, I. (1988) *Biochemistry 27*, 4747−4753.
Jackson, T. A., Lim, M., & Anfinrud, P. A. (1994) *Chem. Phys. 180*, 131−140.
Koch, S., Tang, S. C., Holm, R. H., Frankel, R. B., & Ibers, J. A. (1975) *J. Am. Chem. Soc. 97*, 916−918.
McRee, D. E., Jensen, G. M., Fitzgerald, M. M., Siegel, H. A., & Goodin, D. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12847−12851.
Mintorovitch, J., & Satterlee, J. D. (1988) *Biochemistry 27*, 8045−8050.
Morishima, I., Ogawa, S., Inubushi, T., & Iizuka, T. (1978) *Adv. Biophys. 11*, 217−245.
Newmyer, S. L., & Ortiz de Montellano, P. R. (1995) *J. Biol. Chem. 270*, 19430−19438.
Nordblom, G. D., White, R. E., & Coon, M. J. (1976) *Arch. Biochem. Biophys. 175*, 524−533.
Ortiz de Montellano, P. R., & Catalano, C. E. (1985) *J. Biol. Chem. 260*, 9265−9271.
Palaniappan, V., & Bocian, D. F. (1994) *Biochemistry 33*, 14264−14274.
Peisach, J. (1975) *Ann. N.Y, Acad. Sci. 244*, 187.
Peisach, J., & Blumberg, W. E. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 172−179.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199−8205.
Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122−16130.
Quillin, M. L., Arduini, R. M., Olson, J. S., & Phillips, G. N., Jr. (1993) *J. Mol. Biol. 234*, 140−155.
Ramette, R. W., & Sandford, R. W., Jr. (1965) *J. Am. Chem. Soc. 87*, 5001−5005.
Rao, S. I., Wilks, A., & Ortiz de Montellano, P. R. (1993) *J. Biol. Chem. 268*, 803−809.
Rao, S. I., Wilks, A., Hamberg, M., & Ortiz de Montellano, P. R. (1994) *J. Biol. Chem. 269*, 7210−7216.
Sawaki, Y., & Foote, C. S. (1979) *J. Am. Chem. Soc. 101*, 6292−6296.
Shiro, Y., Iizuka, T., Marubayashi, K., Ogura, T., Kitagawa, T., Balasubramanian, S., & Boxer, S. (1994) *Biochemistry 33*, 14986−14992.
Sun, J., Loehr, T. M., Wilks, A., & Ortiz de Montellano, P. R. (1994) *Biochemistry 33*, 13734−13740.
Traylor, T. G., Koga, N., & Deardurff, L. A. (1985) *J. Am. Chem. Soc. 107*, 6504−6510.
Tsai, R., Yu, C. A. Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., & Beinert, H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1157−1163.
Varadarajan, R., Szabo, A., & Boxer, S. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5681−5684.
Varadarajan, R., Lambright, D. G., & Boxer, S. G. (1989) *Biochemistry 28*, 3771−3781.
Yu, C.-A., Gunsalus, I. C., Katagiri, M., Suhara, K., & Takemori, S. (1974) *J. Biol. Chem. 249*, 94−101.

BI960459Z