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Alteration of Human Myoglobin Proximal Histidine to Cysteine or Tyrosine by Site-Directed Mutagenesis: Characterization and Their Catalytic Activities

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Summary: Two mutant proteins of human myoglobin (Mb) that exhibit altered axial ligations were prepared by site-directed mutagenesis of a cloned gene for human Mb. The normal axial ligand residue, histidine 93(F8), was replaced with cysteine or tyrosine, resulting in H93C or H93Y Mb, respectively. Cysteine or tyrosine coordination to the ferric heme iron is verified by electronic absorption, ^{1}H-NMR, EPR spectra, and redox potentials of Fe^{2+}/Fe^{3+} couple. Their mono-oxygenation activities of styrene are also discussed.

Biological strategy in manipulating hydrogen peroxide or its equivalents is an use of heme enzymes having an anionic axial ligand such as imidazolate, phenolate, or thiolate.[1] While functional importance of the anionic ligands is believed to assist an O-O bond cleavage reaction to form a high-valent iron-oxo complex, details are not clear yet. In the case of P-450, Dawson et al. have claimed a role of the cysteine to be the ligation of thiolate to the heme iron as an electron donor to facilitate heterolytic O-O bond cleavage.[2] The resultant high-valent iron-oxo "compound I" type derivative is the most probable candidate for the intermediate responsible for the catalytic mono-oxygenation. In fact, the redox potential for P-450cam (ferric/ferrous couple) is appreciably lower than that of myoglobin as expected for a more electron-rich metal center.[3] Very similar "push" effects have been also considered for peroxidases and catalases.[4]

Recent in vitro mutagenesis technique enables us to conduct cloning of human myoglobin (Mb) gene and to genetically engineer human Mb by employing the resultant high-level expression of this gene as a fusion protein in *Escherichia coli*.[5] Here we report the preparation, spectroscopic characterization, and the catalytic activity of the novel mutant human Mb whose proximal histidine (93(F8)) is substituted with cysteine or tyrosine, a protein with altered heme-iron ligation analogous to P-450 or catalase.

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MATERIALS AND METHODS

Site-directed Mutagenesis, Protein Preparation and Purification: Human Mb expression vector, pMb3 (pLcIIFXMb) was a gift from Varadarajan and Boxer.[5] The *Bam*HI-*Hin*dIII fragment was cut out from the original expression vector and inserted into the *Bam*HI-*Hin*dIII site of pharge M13mp18 DNA to form M13mp18FXMb. The mutagenesis experiment was carried out as described by Kunkel et al.[6] Probable mutants were sequenced by dideoxy sequencing to ensure that no additional mutations in the Mb coding sequence had occured.[7] The *Bam*HI-*Hin*dIII fragment of M13mp18 FXMb containing the mutant Mb coding region was then ligated to the *Bam*HI-*Hin*dIII fragment from pMb3 expression vector to give the desired expression vector. Protein preparation and purification was performed accordingly to the method described by Varadarajan et al.[8]

Spectroscopy: Electronic absorption spectra of purified proteins in 50 mM sodium phosphate buffer, pH 7.0, were recorded on a Hitachi U-3210 UV / visible spectrophotometer. Concentration of the samples was 10 μM. Electron paramagnetic resonance (EPR) measurements were carried out at 4.2 K and at X-band (9.35GHz) microwave frequency with a home-built EPR spectrometer with a 100-kHz feild modulation by use of a Varian X-band cavity. Hyperfine-shifted ^{1}H-NMR spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer. Concentration of the samples was 1 mM. Measurements of redox potential (Fe^{3+}/Fe^{2+} couple) were performed as previously reported.[9]

Catalytic Mono-oxygenation of Styrene: A solution of the wild-type or mutant Mb (50 μM) and styrene (15 mM) in 50 mM sodium phosphate buffer (pH 7.0) was preincubated at 30°C for 20 min. 1-ml incubations were routinely employed twice for each experimental point in analytical experiments. A preincubated solution of hydrogen peroxide in 50 mM phosphate buffer (1 mM final concentration) was then added to initiate the reaction and the mixtures were incubated at 30°C for 2 min. Cumylalcohol (243 μg / ml) was added as an internal standard at the end of the incubation period to quantitate product formation. The incubation mixtures were then extracted with 5-ml of diethyl ether. The extract was dried over anhydrous sodium sulfate. After evaporation of most of ether, the metabolites were determined by liquid chromatography.

RESULTS AND DISCUSSION

Spectroscopic Characterization: Electronic absorption spectra of met (Fe^{3+}) forms of wild-type and mutant Mbs are shown in Figure 1. Charge transfer bands of the tyrosine substituted mutant (H93Y) at ca. 480 and 600 nm, characteristic of high-spin ferric heme, are also observed in bovine liver catalase, and various types of tyrosine-coordinated Hb M.[10] Similar absorption bands in the range of 470-490nm have been observed in the non heme iron-phenolate proteins and model compounds and have been assigned to the phenolate-Fe(III) $p\pi$-$d\pi$ charge transfer bands.[11] Accordingly, we have assigned a phenolate ligation to heme iron in H93Y Mb. A very similar result was mentioned by Egeberg et al.[12] The electronic absorption spectrum of ferric H93C, the cysteine substituted mutant, is nearly identical to that observed for *d*-camphor-bound ferric P-450cam and synthetic iron(III) porphyrins having thiolate ligands.[13] Especially, the Soret band at 391 nm strongly suggests thiolate-ligation to the heme iron, since most heme proteins except for P-450 have a high-spin Soret band greater than 400 nm. Electronic absorption spectra of cyanomet ($Fe^{3+}CN$), deoxy (Fe^{2+}), oxy ($Fe^{2+}O_2$), and carbon monoxy ($Fe^{2+}CO$) forms of wild-type and the mutant Mbs are summarized in Table 1. Oxy forms of the mutant Mbs were rapidly autoxidized compared to wild-type

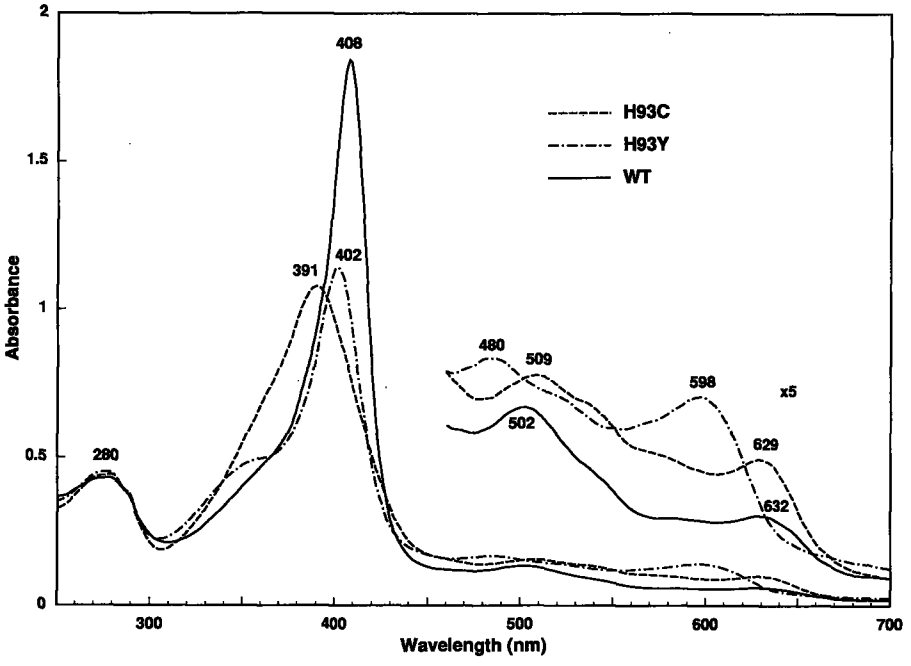


Figure 1. Electronic absorption spectra of ferric wild-type and mutant human Mbs in 50 mM sodium phosphate buffer at pH 7.0 and 20°C.

Table 1

Electronic absorption spectra of various forms of wild-type and mutant Mbs

$Fe^{3+}CN$		
Protein	Soret	visible
H93C	421	550
H93Y	419	545
wild-type	422	541

Fe^{2+}		
Protein	Soret	visible
H93C	428	558
H93Y	427	560
wild-type	433	558

$Fe^{2+}O_2$			
Protein	Soret	β	α
H93C	418	540	575
H93Y	414	540	576
wild-type	417	543	580

$Fe^{2+}CO$			
Protein	Soret	β	α
H93C	420	539	569
H93Y	420	539	567
wild-type	422	541	579

Experimental conditions; in 50 mM sodium phosphate buffer at pH 7.0 and 20°C.

Mb; therefore, spectral measurements were made at 4°C to determine an approximate Soret band for the MbO_2 complexes. In contrast, the mutant Mbs exhibited stable CO complexes under CO atmosphere and at room temperature. Upon reduction of H93C with sodium dithionite under CO atmosphere, the CO complex of H93C was obtained. As the absorption spectrum of the CO complex exhibited a normal MbCO type spectrum (Soret band ; λ_{max} = 420 nm), the proximal thiolate would not ligate at the ferrous state likewise cytochrome P-420. However, under an aerobic condition, the CO form of H93C was autooxidized to give ferric H93C whose absorption spectrum was identical to that of the initial ferric form, and this result indicates that the H93C mutant is not denaturated in the reduced form.

Figure 2 presents hyperfine-shifted ^{1}H-NMR spectra of ferric form of camphor-bound P-450cam and wild-type and the mutant Mbs at room temperature (23°C). The intense

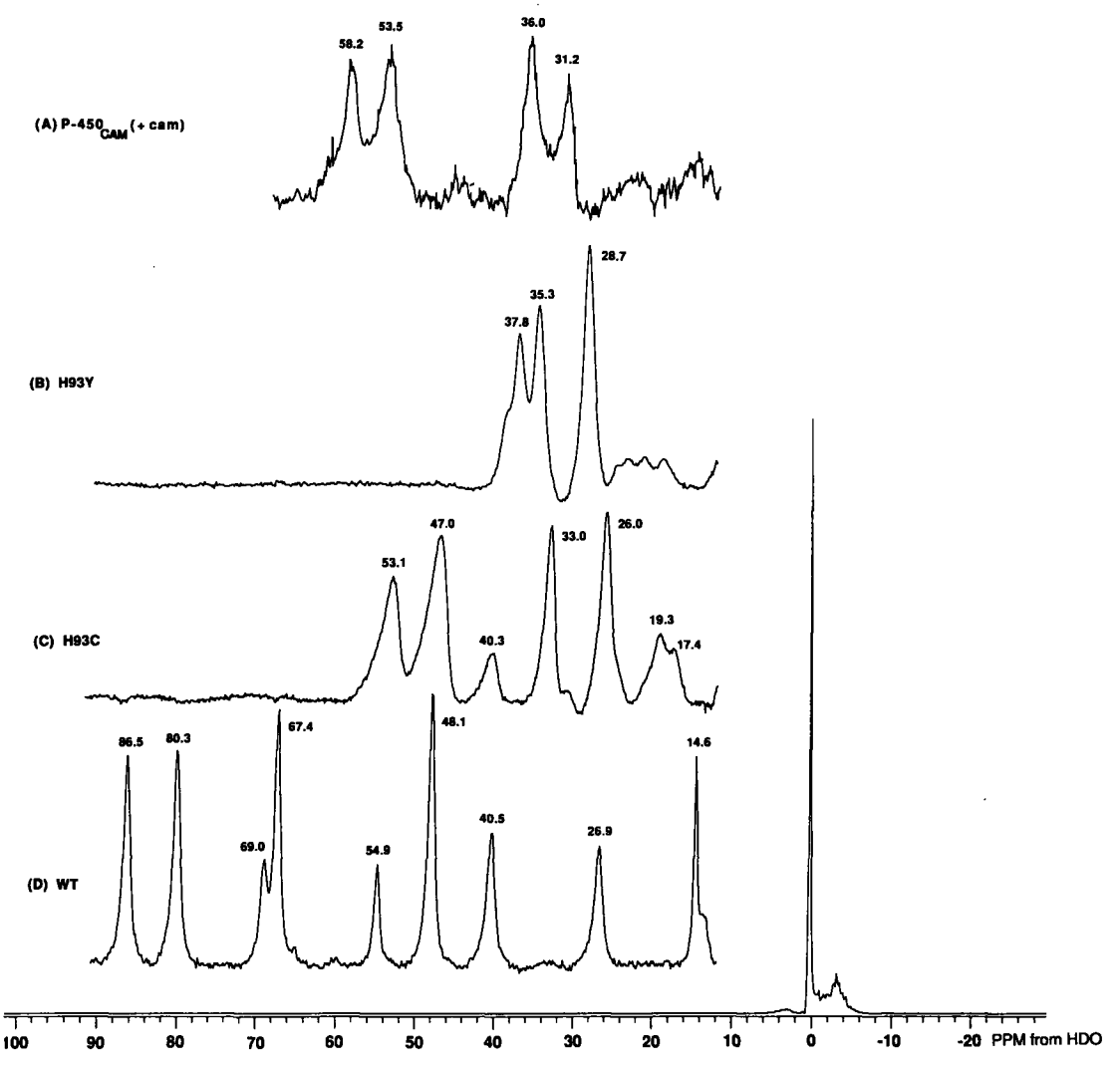


Figure 2. Hyperfine-shifted ^{1}H-NMR spectra of (A) camphor-bound ferric P-450cam and mutant ((B) H93Y, (C) H93C) and (D) wild-type human Mbs in 50mM sodium phosphate buffer at pD 7.0 and 23°C.

peaks for these spectra are due to heme methyl groups. For wild-type Mb, the hyperfine-shifted four heme peripheral methyl peaks are observed at 86.5, 80.3, 67.4, and 48.1 ppm far downfield from the HDO resonance,[14] while corresponding signals are located at 53.1, 47.0, 33.0, and 26.0 ppm for H93C Mb, and at 37.8, 35.3, and 28.7 ppm for H93Y Mb. As shown in Figure 2(A) and 2(C), the spectrum of ferric H93C is nearly identical with camphor-bound ferric P-450cam. While ferric H93Y shows less hyperfine-shifted signals than those of bovine liver catalase, similarity of the spectral characteristics is evident.[15] These results clearly indicate that the electronic structure of the mutants resembles that of corresponding heme enzymes.

The electron paramagnetic resonance (EPR) of the ferric form of the mutant Mbs was examined. H93Y Mb exhibits multiple g values of 6.61, 5.97, 5.22, and 2.00 suggestive of at least two ligation states. These results appear to agree with the reported g values of the bovine liver catalase and mutant sperm whale Mb whose proximal His is altered to Tyr.[12,16] H93C Mb shows a mixture of high- and low-spin species at 4.2 K (high-spin; g = 8.41, 3.17, 1.59 low-spin; g = 2.35, 2.22, 1.95) just as reported for ferric P-450cam in the existence of *d*-camphor at 15 K,[17] suggesting that H93C is in a thermal spin equilibrium or is ligated by an internal ligand (such as distal histidine) by the freezing effect. Appearance of the low-spin state upon lowering temperature was also observed in electronic absorption spectroscopy, i.e, the intensity of the 629-nm band corresponding to ferric high-spin heme is remarkably reduced at -60°C (in a frozen matrix) as a reversible process. These results indicate that low-spin species appears at low temperature, while high-spin species is predominant at room temperature. The g values of H93C at 8.41 and 3.17 are very rhombic among various heme proteins, and strongly suggests a thiolate ligation. Palmer et al. showed percent rhombicity of several native heme proteins in its ferric high-spin state defined from g values in the g = 6 region.[18] H93C and H93Y mutants display high-rhombicities (% rhombicity: H93C; 32.8 %, H93Y; 8.7 %) nearly identical with that reported for P-450cam (26.0 %) and bovine liver catalase (type I; 7.5 %, type II; 11.7 %), respectively.

Combining these spectroscopic data, it is verified that cysteine or tyrosine is coordinated to ferric iron of H93C or H93Y Mb, respectively, and that ferric heme iron of these mutants is predominantly in high-spin state at room temperature, while H93C is partially in low-spin state at 4.2 K.

Redox Potential Measurements of Ferric/Ferrous Couple: Redox potential measurements (Fe^{3+}/Fe^{2+} couple) for the wild-type and the mutants were also examined. A preliminary result shows that the redox potentials of the mutants were extremely lower (H93C : -210 mV, H93Y : -190 mV) than that of wild-type Mb (+50 mV). In Table 2, redox potentials of various heme proteins are compiled. Heme enzymes such as P-450cam, horseradish peroxidase, and catalase have their redox potentials lower than that of human Mb, and this result has been interpreted by more-electron rich metal center due to push effect of the anionic axial ligands.[3,4] Very low redox potentials of the Mb mutants compared to wild-type Mb strongly suggest anionic thiolate or phenolate ligation to the heme iron in H93C or H93Y Mbs, respectively.

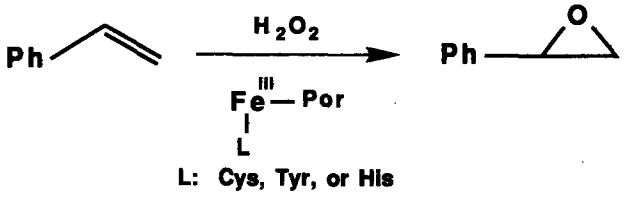
Table 2. Redox potentials (Fe^{3+}/Fe^{2+} couple) of various heme proteins

protein	redox potential (mV)	ref.
P-450cam (low spin)	-170	3a
P-450cam (high spin)	-270	3a
horse radish peroxidase	-250	9
catalase	<-500	4
sperm whale Mb	+55	3b
human Mb (wild-type)	+50	this study
H93C	-230	this study
H93Y	-190	this study

Catalytic Mono-oxygenation of Styrene: The catalytic mono-oxygenase activity of P-450 has been attributed to the formation of a two electron oxidized iron-oxo "compound I" type species, according to the heterolytic O-O bond cleavage.[2)] While wild-type Mb is known to react with H_2O_2 to yield "compound II" intermediate, reported mono-oxygenase activity of Mb is lower than that of P-450.[19)] Thus, we have studied the effect of replacement of the imidazole ligand to the thiolate or phenolate on the catalytic mono-oxygenase activity of Mb by employing the peroxide shunt reaction.[2a)] As a typical P-450 reaction, epoxidation of styrene was examined. (Scheme 1)

Initial reaction rates of H93C (4.6 x 10^{-2} turnover no./min) and H93Y (9.5 x 10^{-3} turnover no./min) were 5.1 and 1.1 times faster than that of wild type human Mb (9.0 x 10^{-3} turnover no./min), respectively. Epoxidation of styrene by using hydrogen peroxide and sperm whale, horse Mb or bovine Hb has been reported by Ortiz de Montellano and co-workers,[19)] and our result of reaction rate for human wild type Mb roughly agrees with their results for bovine Hb.

The reaction rates of the mutants were slowly decreased with concomitant decrease of the Soret band absorption, probably due to oxidation of anionic ligand and/or heme degradation. The addition of H_2O_2 to the reaction solution containing wild type human Mb caused immediate formation of compound II, whereas any attempts to detect higher-valent intermediates were failed in the case of the mutant Mbs under the same condition. Apparently, introduction of thiolate or phenolate ligand to Mb has altered its enzymic activity. Details of anionic ligand effect on the mono-oxygenase and catalase activities are under investigation in this laboratory.



Scheme 1

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REFERENCES

(1) (a) Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Eriksson, B., Xuong, N.-H., Yonetani, T., and Kraut, J. (1980) *J. Biol. Chem.* **255**, 575-580.
(b) Murthy, M. R. N., Reid, T. J. III, Sicignano, A., Tanaka, N., and Rossmann, M. G. (1981) *J. Mol. Biol.* **152**, 465-499.
(c) Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner G. C., and Kraut, J. (1985) *J. Biol. Chem.* **260**, 16122-16130.

(2) (a) Dawson, J. H., and Sono, M. (1987) *Chem. Rev.* **87**, 1255-1276.
(b) Dawson, J. H., Holm, R. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., and Tang, S. C. (1976) *J. Am. Chem. Soc.* **98**, 3707-3709.

(3) (a) Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P., and Münk, E. (1974) in *Molecular Mechanisms of Oxygen Activation* p. 559, Academic Press, New York.
(b) Antonini, E., and Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands*, p. 330, North-Holland Publishing Co., Amsterdam and London.

(4) Williams, R. J. P. (1974) in *Iron in Biochemistry and Medicine*, (Jacobs, A., and Worwood, M., Eds.) p. 183-219, Academic Press, New York.

(5) Varadarajan, R., Szabo, A., and Boxer, S. G. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5681-5684.

(6) Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488-492.

(7) Sanger, F., Miklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

(8) Varadarajan, R., Lambright, D. G., and Boxer, S. G. (1989) *Biochemistry* **28**, 3771-3781.

(9) Yamada, H., Makino, R., and Yamazaki, I. (1975) *Arch. Biochem. Biophys.* **169**, 344-353.

(10) (a) Nicholls, P., and Schonbaum, G. R. (1963) in *The Enzyme* (Boyer, P. D., Lardy, H., and Myrbëck, K., Eds.) Vol. 8, p. 147-225, Academic Press, New York.
(b) Nagai, M., and Yoneyama, Y. (1983) *J. Biol. Chem.* **258**, 14379-14384.
(c) Nagai, M., Yoneyama, Y., and Kitagawa, T. (1989) *Biochemistry* **28**, 2418-2422.

(11) Que, L., Jr. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, p. 491, Wiley, New York.

(12) Egeberg, K. D., Springer, B. A., Martinis, S. A., Sliger, S. G., Morikis, D., Champion, P. M. (1990) *Biochemistry* **29**, 9783-9791.

(13) (a) Yu, C. A., Gunsalus, I. C., Katagiri, M., Suhara, K., and Takemori, S. (1974) *J. Biol. Chem.* **249**, 94-101.
(b) Tang, S. C., Koch, S., Papaefthymiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A., and Holm, R. H. (1976) *J. Am. Chem. Soc.* **98**, 2414-2434.

(14) Morishima, I., Ogawa, S., Inubushi, T., and Iizuka, T. (1978) *Adv. in Biophys.* **11**, 217-245.

(15) Morishima, I., and Ogawa, S. (1982) in *Oxidases and Related Redox Systems* (King, T. E. et al., Eds.) p. 599, Pergamon Press, Oxford and New York.

(16) (a) Torii, K., and Ogura, Y. (1968) *J. Biochem.* **64**, 171-179.
(b) Hayashi, A., Suzuki, T., Imai, K., Morimoto, H., and Watari, H. (1969) *Biochim. Biophys. Acta.* **194**, 6-15.

(17) Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H. (1970) *Proc. Natl. Acad. Sci. USA* **66**, 1157-1163.

(18) Palmer, G. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. IV, p. 328, Academic Press, New York.

(19) Ortiz de Montellano, P. R. and Catalano, C. E. (1985) *J. Biol. Chem.* **260**, 9265-9271.