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Electronic Control of Ligand-Binding Preference of a Myoglobin Mutant

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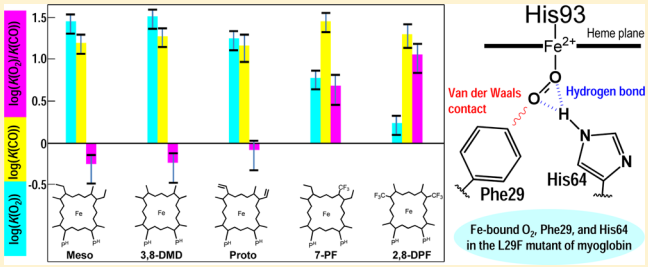
S *Supporting Information*

ABSTRACT: The L29F mutant of sperm whale myoglobin (Mb), where the leucine 29 residue was replaced by phenylalanine (Phe), was shown to exhibit remarkably high affinity to oxygen (O_2), possibly due to stabilization of the heme Fe atom-bound O_2 in the mutant protein through a proposed unique electrostatic interaction with the introduced Phe29, in addition to well-known hydrogen bonding with His64 [Carver, T. E.; Brantley, R. E.; Singleton, E. W.; Arduini, R. M.; Quillin, M. L.; Phillips, G. N., Jr.; Olson, J. S. *J. Biol. Chem.*, **1992**, 267, 14443−14450]. We analyzed the O_2 and carbon monoxide (CO) binding properties of the L29F mutant protein reconstituted with chemically modified heme cofactors possessing a heme Fe atom with various electron densities, to determine the effect of a change in the electron density of the heme Fe atom (ρ_{Fe}) on the O_2 versus CO discrimination. The study demonstrated that the preferential binding of O_2 over CO by the protein was achieved through increasing ρ_{Fe}, and the ordinary ligand-binding preference, that is, the preferential binding of CO over O_2, by the protein was achieved through decreasing ρ_{Fe}. Thus, the O_2 and CO binding preferences of the L29F mutant protein could be controlled through electronic modulation of intrinsic heme Fe reactivity through a change in ρ_{Fe}. The present study highlighted the significance of the tuning of the intrinsic heme Fe reactivity through the heme electronic structure in functional regulation of Mb.



INTRODUCTION

Myoglobin (Mb) is a hemoprotein found in muscle cells, where it is responsible for the storage of dioxygen (O_2) in energy metabolism.[1] In addition to O_2, carbon monoxide (CO) is also reversibly bound to a ferrous heme Fe atom (Fe(II)) in Mb. As an O_2 storage protein, Mb must discriminate O_2 from the toxic ligand CO ubiquitously produced from a variety of sources in biological systems.[2] The ligand-binding affinity of unencumbered model heme Fe(II) complexes is $\sim 2 \times 10^4$ times higher for CO than for O_2, but this ratio is considerably reduced in the proteins.[3,4] Ever since the three-dimensional structure of Mb was determined as the first protein crystal structure,[5] elucidation of the mechanism responsible for the discrimination between O_2 and CO has attracted many researchers' interest.[1−4,6,7] The distal histidine residue (His64) has been shown to play a central role in the control of O_2 versus CO discrimination in Mb.[1−4,6,7] His64 forms a hydrogen bond with the Fe(II)-bound ligand to stabilize its bound state,[8−15] and the hydrogen bond is formed more favorably with Fe(II)-bound O_2 than with Fe(II)-bound CO.[16,17] We recently revealed that the O_2 affinity of Mb is greatly affected by the electron density of the heme Fe atom (ρ_{Fe}), whereas the CO affinity is almost independent of ρ_{Fe}.[18,19] Thus, even in the absence of His64, the O_2 versus CO discrimination by the protein can be controlled through electronic tuning of the intrinsic heme Fe reactivity through ρ_{Fe}. ρ_{Fe} has been shown to be semiquantitatively manifested in either the equilibrium constant pK_a of the so-called "acid−alkaline transition" in the met-form of the protein,[18] that is, Fe(III), or the stretching frequency of Fe(II)-bound CO (ν_{CO}) in the protein,[19] and comparison between pK_a and ν_{CO} of the protein reconstituted with a variety of chemically modified heme cofactors demonstrated that the

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Table 1. O_2 and CO Binding Parameters for the L29F Mutant Proteins, Native Mbs, and H64L Mutant Proteins at pH 7.40 and 20 °C, and *Paramphistomum epiclitum* Hemoglobin (PeHb) at pH 7.0 and 25 °C

		O_2 binding			CO binding			
heme	protein	$k_{on}(O_2)$ (μM^{-1} s^{-1})	$k_{off}(O_2)$ (s^{-1})	$K(O_2)$ (μM^{-1})	$k_{on}(CO)$ (μM^{-1} s^{-1})	$k_{off}(CO)$ (s^{-1})	$K(CO)$ (μM^{-1})	M[a]
Meso	L29F	21 ± 4	0.78 ± 0.2	27 ± 8	0.19 ± 0.04	0.013 ± 0.003	15 ± 4	0.56 ± 0.2
	Mb[b]	8.2 ± 1.6	5.7 ± 1.1	1.5 ± 0.4	0.38 ± 0.07	0.048 ± 0.009	7.9 ± 2.4	5.5 ± 2.2
	H64L[c]	222 ± 67	2300 ± 970	0.097 ± 0.069	29 ± 8.7	0.25 ± 0.075	116 ± 48.7	1200 ± 990
3,8-DMD	L29F	25 ± 5	0.80 ± 0.2	31 ± 9	0.21 ± 0.04	0.012 ± 0.002	18 ± 5	0.58 ± 0.2
	Mb[d]	12 ± 3	9.7 ± 3	1.3 ± 0.3	0.16 ± 0.07	0.024 ± 0.007	6.7 ± 1.7	5.1 ± 2.1
	H64L[c]	292 ± 88	1900 ± 800	0.15 ± 0.11	32 ± 9.6	0.23 ± 0.069	139 ± 58.4	930 ± 770
7-PF	L29F	22 ± 4	3.8 ± 0.8	5.8 ± 2	0.38 ± 0.08	0.014 ± 0.003[e]	27 ± 8	4.7 ± 2
	Mb[b]	8.3 ± 1.6	17 ± 3	0.5 ± 0.1	0.32 ± 0.06	0.032 ± 0.006	10 ± 3	21 ± 8
	H64L[c]	212 ± 64	5700 ± 2400	0.037 ± 0.026	21 ± 6.3	0.052 ± 0.016[f]	580 ± 244	11 000 ± 9100
2,8-DPF	L29F	31 ± 6	18 ± 4	1.7 ± 0.5	0.33 ± 0.07	0.017 ± 0.003	19 ± 6	11 ± 4
	Mb[b]	16 ± 3	110 ± 22	0.15 ± 0.03	0.69 ± 0.13	0.036 ± 0.007	19 ± 6	132 ± 53
	H64L[c]	305 ± 92	31 000 ± 13 000	0.0098 ± 0.007	29 ± 8.7	0.053 ± 0.016	547 ± 230	56 000 ± 46 000
Proto	L29F	26 ± 5	1.5 ± 0.3	17 ± 5	0.23 ± 0.05	0.016 ± 0.003	14 ± 5	0.82 ± 0.3
	Mb[b]	14 ± 3	12 ± 2	1.2 ± 0.3	0.51 ± 0.06	0.019 ± 0.005	27 ± 8	23 ± 9
	H64L[c]	228 ± 68	3200 ± 1300	0.071 ± 0.05	30 ± 9.0	0.061 ± 0.018	492 ± 207	6900 ± 5700
Proto	PeHb[g]	90	0.07	1300	25	0.1	250	0.19

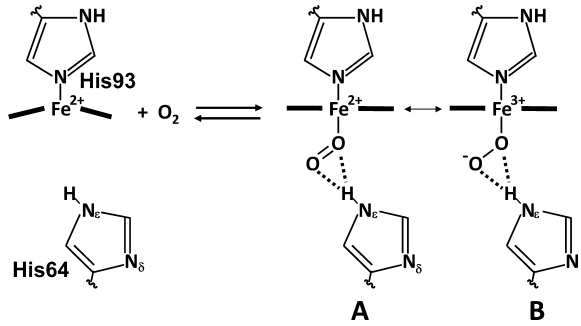
[a] M represents the quantity $K(CO)/K(O_2)$. [b] Taken from ref 18. [c] Taken from ref 41. [d] Taken from ref 19. [e] Calculated from a weighted average of fitted parameters because the time course was biphasic, and the sum of two exponentials provided a better fit to the data. [f] Biphasic time evolution of CO association was fitted by the sum of two exponentials, and the value closer to those of the other H64L mutant proteins is indicated; the other one is 5.69 ± 1.7. [g] Taken from ref 43.

effects of heme modifications on ρ_{Fe} are essentially independent of the oxidation state of the heme Fe atom.[19]

In this study, we attempted to control the O_2 versus CO ligand-binding preference of the L29F mutant protein of sperm whale Mb, in which the leucine 29 (Leu29) residue is replaced by phenylalanine (Phe), through alteration of ρ_{Fe}. Leu29 in Mb is located close to His64, although its isobutyl side chain is not in van der Waals contact with either the Fe-bound ligand or the heme cofactor. Leu29 is highly conserved in Mbs and hemoglobins (Hbs), and it has been shown to play an important role in control of their ligand-binding properties.[20] The L29F mutant protein exhibits high O_2 affinity (Table 1).[20,21] An X-ray crystallographic study of the L29F mutant revealed that, in addition to the above-mentioned hydrogen bonding with His64, the coordination of O_2 to Fe(II) in the mutant is also stabilized through favorable electrostatic interaction of the polar Fe(II)-bound O_2 with the multipole of the phenyl ring of the introduced Phe29.[20] With the aid of both His64 and Phe29, the rate constant for O_2 dissociation ($k_{off}(O_2)$) of the L29F mutant decreased by a factor of ~1/8 compared with that of the native Mb.[20] In addition, the rate constant for O_2 association ($k_{on}(O_2)$) of the mutant protein was greater by a factor of ~2 than that of the native Mb, whereas the rate constants for CO association and dissociation ($k_{on}(CO)$ and $k_{off}(CO)$, respectively) of the mutant protein were similar to the corresponding values of the native one.[20]

Our strategy is based on the previous findings that $k_{off}(O_2)$ increases (decreases) with decreasing (increasing) ρ_{Fe}, whereas the other kinetic values, that is, $k_{on}(O_2)$, $k_{on}(CO)$, and $k_{off}(CO)$, are essentially independent of ρ_{Fe}.[18,19] The relationship between ρ_{Fe} and $k_{off}(O_2)$ could be interpreted in terms of the effect of a change in ρ_{Fe} on the resonance process between the $Fe^{2+}-O_2$ and $Fe^{3+}-O_2^-$-like species (Scheme 1),[8,22] and, for example, a decrease in the ρ_{Fe} stabilizes the $Fe^{2+}-O_2$ species over the $Fe^{3+}-O_2^-$-like one, resulting in an increase in $k_{off}(O_2)$. To greatly alter ρ_{Fe}, we substituted strongly electron-withdrawing trifluoromethyl (CF_3) group(s), as peripheral side chain(s) of the heme cofactor, to prepare 13,17-bis(2-carboxylatoethyl)-3,8-diethyl-2,12,18-trimethyl-7-trifluoromethylporphyrinatoiron(III)[23] (7-PF) and 13,17-bis(2-carboxylatoethyl)-3,7-diethyl-12,18-trimethyl-2,8-ditrifluoromethylporphyrinatoiron(III)[18] (2,8-DPF) (Figure 1). In addition, mesoheme (Meso) and 3,8-dimethyldeuteroporphyrinatoiron(III)[24,25] (3,8-DMD) were used as counterparts of 7-PF and 2,8-DPF, respectively. The apoprotein of the L29F mutant protein was reconstituted with Meso, 3,8-DMD, 7-PF, and 2,8-DPF to yield mutant proteins possessing the corresponding heme cofactors, that is, L29F(Meso), L29F(3,8-DMD), L29F(7-PF), and L29F(2,8-DPF), respectively. Pairwise comparison between L29F(Meso) and L29F(7-PF), and between L29F(3,8-DMD) and L29F(2,8-DPF), reveals the structural and functional consequences of the substitution of one and two CF_3 groups, respectively. As expected from the previous findings, the preferential binding of O_2 over CO was observed for L29F(Meso) and L29F(3,8-

Scheme 1. Oxygenation of Mb[a]



[a] The binding of O_2 to the heme Fe atom is stabilized by the hydrogen bonding between the Fe^{2+}-bound O_2 and His64.[6,10,16,17] Structure (B) of the oxy form is only a proposed one.[8,22].

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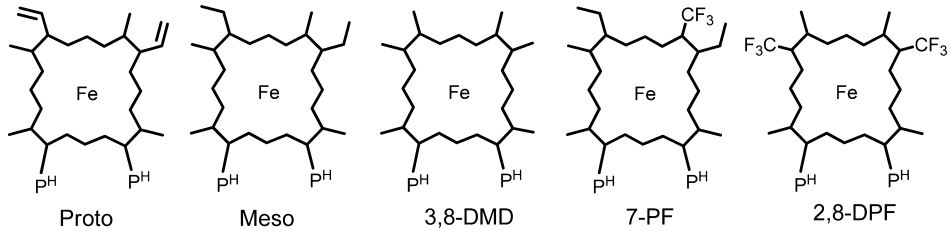

Figure 1. Schematic representation of the structures of the heme cofactors used in this study, that is, protoheme (Proto), mesoheme (Meso), 3,8-dimethyldeuteroporphyrinatoiron(III)[24,25] (3,8-DMD), 13,17-bis(2-carboxylatoethyl)-3,8-diethyl-2,12,18-trimethyl-7-trifluoromethylporphyrinatoiron(III)[23] (7-PF), and 13,17-bis(2-carboxylatoethyl)-3,7-diethyl-12,18-trimethyl- 2,8-ditrifluoromethylporphyrinatoiron(III)[18] (2,8-DPF). Abbreviation: P^H represents $-CH_2CH_2COOH$.

DMD), whereas L29F(7-PF) and L29F(2,8-DPF) exhibited the ordinary ligand-binding preference. Thus, the O_2 versus CO ligand-binding preference of the L29F Mb mutant protein could be controlled through a change in ρ_{Fe}. These findings confirmed the significant effect of a change in the heme electronic structure on Mb function.

MATERIALS AND METHODS

Materials and Protein Samples. All reagents and chemicals were obtained from commercial sources and were used as received. The expression and purification of the L29F mutant protein (L29F(Proto)) of sperm whale Mb were carried out according to the methods described by Carver et al.[20] Mesoheme (Meso) was purchased from Frontier Scientific Co. 2,8-DPF,[18] 7-PF,[23] and 3,8-DMD[24,25] were synthesized as previously described. The apoprotein of the L29F mutant protein was prepared at 4 °C according to Teale's procedure,[26] and reconstituted L29F mutant proteins were prepared by slow addition of a synthetic heme cofactor to the apoprotein in 50 mM potassium phosphate buffer, pH 7.0, at 4 °C.[18] The reconstituted proteins were kept at 4 °C for about 48 h for equilibration of well-known heme orientational disorder (see below).[27] To prepare the CO and O_2 forms of the L29F mutant proteins, the proteins were reduced by adding $Na_2S_2O_4$ (Nacalai Chemicals Ltd.) in the presence of CO gas (Japan Air Gases) and in air, respectively. The stability of the O_2 form of the L29F mutant protein was ensured by extremely slow autoxidation.[20] Excess reducing agent was removed by passage through a Sephadex G-10 (Sigma-Aldrich Co.) column equilibrated with an appropriate buffer solution. The met-cyano forms of the L29F mutant proteins were prepared by adding KCN (Nacalai Chemicals Ltd.) to the met-forms of the proteins obtained through oxidation of the proteins using $K_3Fe(CN)_6$ (Nacalai Chemicals Ltd.). For NMR samples, the proteins were concentrated to ~1 mM in 0.1 M phosphate buffer at pH 7.40, and then 10% ^2H_2O was added to the protein solutions. The pH of each sample was measured with a Horiba F-22 pH meter equipped with a Horiba type 6069-10c electrode.

NMR Spectroscopy. ^1H NMR spectra of the met-cyano forms of the L29F mutant proteins were recorded on a Bruker AVANCE-600 spectrometer operating at the ^1H frequency of 600 MHz. Typical ^1H NMR spectra consisted of about 20 000 transients with a 100 kHz spectral width and 16 000 data points. The signal-to-noise ratio of the spectra was improved by apodization, which introduced ~10 Hz line broadening. The chemical shifts of ^1H NMR spectra are given in ppm downfield from the residual $^1H^2HO$ peak at 4.75 ppm, as a secondary reference.

Kinetic Measurements of O_2 and CO Binding. Kinetic measurements of the proteins were carried out in 100 mM phosphate buffer, pH 7.40, at 20 °C using the procedure reported previously.[1,28−30] The O_2 associations for L29F(Proto), L29F(Meso), L29F(3,8-DMD), L29F(7-PF), and L29F(2,8-DPF) were characterized through analysis of the time evolution of the absorbance at 404, 410, 400, 397, and 410 nm, respectively, after photolysis of their oxy forms, under air, using a 5 ns pulse Nd:YAG laser (532 nm, Continuum Surelite II). The probe light (Xe-lamp) was passed through a monochromator before a sample with adjustment to a suitable wavelength in each experiment. The fitting of the time evolution of the absorbance to the first-order rate equation yielded a pseudo-first-order rate constant for O_2 association ($k_{obs}(O_2)$), which can be expressed in terms of $k_{on}(O_2)$ and $k_{off}(O_2)$ as $k_{obs}(O_2) = k_{on}(O_2) \times [O_2] + k_{off}(O_2)$. Since $k_{off}(O_2) \ll k_{on}(O_2) \times [O_2]$, $k_{on}(O_2)$ can be determined from the pseudo-first-order rate constant for O_2 association ($k_{obs}(O_2)$) through the equation $k_{obs}(O_2) = k_{on}(O_2) \times [O_2]$, where $[O_2] = 2.52 \times 10^{-4}$ M. Pseudo-first-order rate constants for O_2 dissociation ($k_{off}(O_2)$) for L29F(Proto), L29F(Meso), L29F(3,8-DMD), L29F(7-PF), and L29F(2,8-DPF) were also measured through analysis of the time evolution of the absorbance at 570, 565, 567, 564, and 560 nm, respectively, after rapid mixing of their oxy forms with excess sodium dithionite using a UNISOKU RSP-601 stopped-flow apparatus.

The CO associations for L29F(Proto), L29F(Meso), L29F(3,8-DMD), L29F(7-PF), and L29F(2,8-DPF) were similarly measured through analysis of the time evolution of the absorbance at 420, 410, 410, 409, and 415 nm, respectively, after photolysis of their CO forms under 1 atm of CO, that is, $[CO] = 9.85 \times 10^{-4}$ M. Since $k_{off}(CO) \ll k_{on}(CO) \times [CO]$, $k_{on}(CO)$ can be determined from the pseudo-first-order rate constant for CO association ($k_{obs}(CO)$) through the equation $k_{obs}(CO) = k_{on}(CO) \times [CO]$.

The CO dissociation for the mutant proteins was characterized utilizing the following reactions, which comprise the displacement of Fe-bound CO and the oxidation of heme iron by $K_3Fe(CN)_6$.[28,30]

$$Mb_{Fe(II)}(CO) \underset{k_{on}(CO)}{\overset{k_{off}(CO)}{\rightleftharpoons}} Mb_{Fe(II)} + CO \xrightarrow{k_{ox}} Mb_{Fe(III)}(H_2O)$$

where $Mb_{Fe(II)}$ and $Mb_{Fe(III)}(H_2O)$ represent the deoxy and met-aquo forms of the protein, respectively, and k_{ox} is the rate constant for the oxidation of the heme iron. Under the experimental conditions of high $[CO]$ and $[K_3Fe(CN)_6]$, where a steady-state assumption can be made for $[Mb_{Fe(II)}]$, the pseudo-first-order rate constant for the oxidation of the proteins ($k_{obs}(ox)$) can be expressed in terms of a pseudo-first-order rate constant for CO dissociation ($k_{off}(CO)$), $[K_3Fe(CN)_6]$, and a constant c, as $k_{obs}(ox) = k_{off}(CO) \times [K_3Fe(CN)_6]/(c + [K_3Fe(CN)_6])$. The saturated value in plots of $k_{obs}(ox)$ against $[K_3Fe(CN)_6]$ affords $k_{off}(CO)$. The $k_{obs}(ox)$ values for L29F(Proto), L29F(Meso), L29F(3,8-DMD), L29F(7-PF), and L29F(2,8-DPF) with various $[K_3Fe(CN)_6]$ were determined through analysis of the time evolution of the absorbance at 530, 519, 520, 523, and 525 nm, respectively. The probe light (Xe lamp) was passed through a monochromator before a sample with adjustment to a suitable wavelength in each experiment. Prior to kinetic measurements, it was confirmed in preliminary experiments using a photodiode array detector that the reaction of a ligand-bound Mb with $K_3Fe(CN)_6$ really yielded the corresponding met-aquo form without accumulation of any intermediates.

Resonance Raman Spectroscopy. Resonance Raman scattering was excited at 413.1 nm with a Kr^+ laser (Spectra Physics, BeamLok 2060), dispersed with a polychromator (Ritsu Oyo Kogaku MC-100DG, equipped with 1200 grooves/mm grating), and detected with a liquid nitrogen-cooled charge coupled device (CCD) detector (LN/CCD-1100-PB/VISAR/1, Roper Scientific).[31] The protein concentrations were approximately 40 μM in 100 mM potassium phosphate

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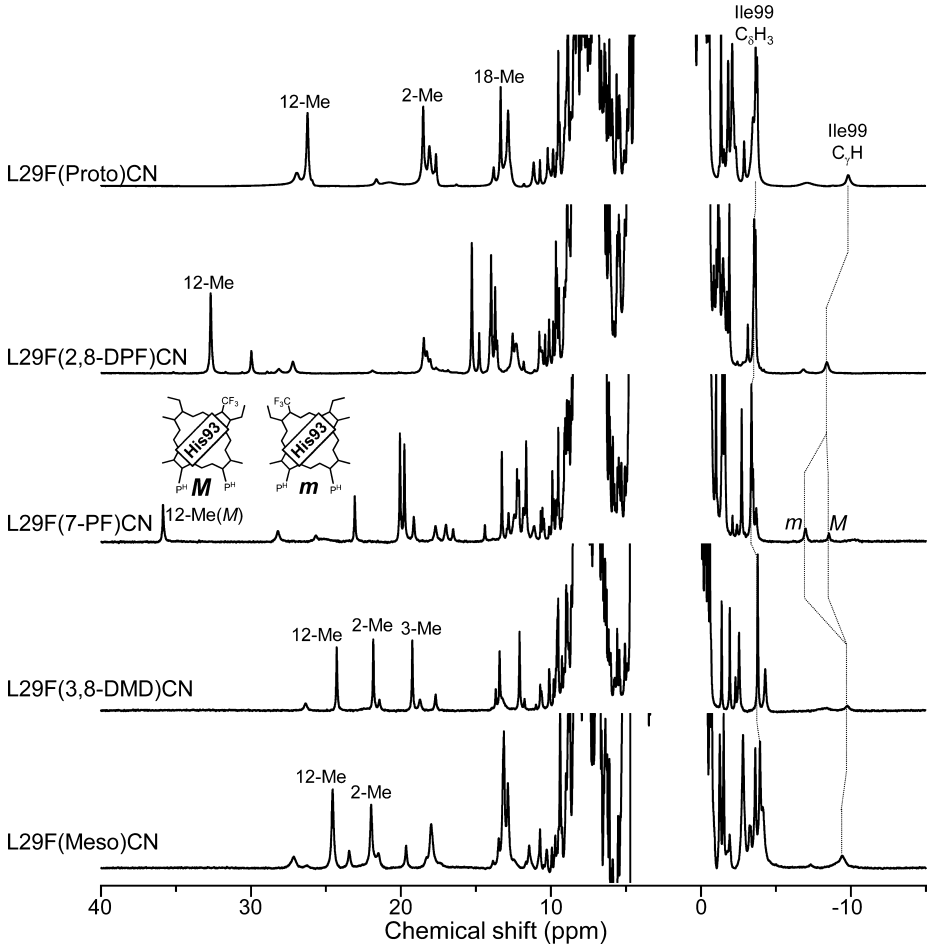

Figure 2. 600 MHz ^{1}H NMR spectra of L29F(Meso)CN, L29F(3,8-DMD)CN, L29F(7-PF)CN, L29F(2,8-DPF)CN, and L29F(Proto)CN at pH 7.40 in 90% H_2O/10% ^2H_2O at 25 °C. The assignments of Ile99 proton signals and the tentative ones of heme methyl proton signals are given with the spectra, and the *M* and *m* forms in the inset represent the two different orientations of 7-PF, relative to the protein.[27] The Ile99 Cγ' H proton signals of the mutant proteins are connected by a broken line.

buffer, pH 7.40. Raman shifts were calibrated with indene as a frequency standard. The positions of the bands were determined through fitting with Voigt profiles, which comprise convolutions of Gaussian and Lorentzian functions,[32] and the accuracy of the peak positions of well-defined Raman bands was ±1 cm^{-1}.

RESULTS

^{1}H NMR Spectra of Met-Cyano Forms of the L29F Mutant Proteins. 600 MHz ^{1}H NMR spectra of the met-cyano forms of the L29F mutant proteins, that is, L29F(Proto)CN, L29F(Meso)CN, L29F(3,8-DMD)CN, L29F(7-PF)CN, and L29F(2,8-DPF)CN, are shown in Figure 2. The paramagnetically shifted NMR signals due to heme side chain protons and amino acid protons in close proximity to the heme in a protein have been shown to be quite sensitive to the heme electronic structure and the heme active site structure, respectively.[33−35] The shift pattern of the paramagnetically shifted ^{1}H NMR signals of L29F(Proto)CN was essentially identical to that previously reported by Zhao et al.[36] The similarity in the shifts of the isoleucine 99 (Ile99) $C_\delta H_3$ and $C_\gamma H$ proton signals, resolved at ∼−4 and ∼−10 ppm, respectively, among the mutant proteins, supported the idea that the orientations of the heme cofactors with respect to the polypeptide chains in these mutant proteins are similar to each other. In addition, the observation of two sets of heme methyl proton signals in the spectrum of L29F(7-PF)CN, the major form (*M*)/minor form (*m*) ratio being 1:2 is due to the presence of well-known heme orientational isomers,[27] as depicted in the inset in Figure 2.

Effects of Heme Cofactor Modifications on Functional Properties of the L29F Mutant Proteins. The kinetic parameters obtained for L29F(Proto) at pH 7.40 and 20 °C, that is, $k_{on}(O_2)$ = 26 ± 5 μM^{-1} s^{-1}, $k_{off}(O_2)$ = 1.5 ± 0.3 s^{-1}, $k_{on}(CO)$ = 0.23 ± 0.05 μM^{-1} s^{-1}, and $k_{off}(CO)$ = 0.016 ± 0.003 s^{-1} (Table 1 and Figures SI1−SI4 in the Supporting Information), agreed well with those reported previously for the protein at pH 7.0 and 20 °C,[20] except for $k_{off}(CO)$, which was larger by a factor of ∼3 compared with the previous one, that is, $k_{on}(O_2)$ = 21 ± 2 μM^{-1} s^{-1}, $k_{off}(O_2)$ = 1.4 ± 0.4 s^{-1}, $k_{on}(CO)$ = 0.22 ± 0.01 μM^{-1} s^{-1}, and $k_{off}(CO)$ = 0.0060 s^{-1}. L29F(Meso) and L29F(3,8-DMD) exhibited $k_{off}(O_2)$ values smaller by a factor of ∼1/2 relative to that of L29F(Proto), that is, 0.78 ± 0.2 s^{-1} and 0.80 ± 0.2 s^{-1} for L29F(Meso) and L29F(3,8-DMD), respectively, whereas the other kinetic values, that is, $k_{on}(O_2)$, $k_{on}(CO)$, and $k_{off}(CO)$, of L29F(Meso) and L29F(3,8-DMD) were similar to the corresponding ones of L29F(Proto) (Table 1). As a result, the O_2 affinities, that is, $K(O_2)$ values, of L29F(Meso) and L29F(3,8-DMD) were increased by a factor of ∼2 relative to that of L29F(Proto), that is, 27 ± 8 μM^{-1} and 31 ± 9 μM^{-1} for L29F(Meso) and L29F(3,8-DMD), respectively, and hence the O_2 affinities of

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these two mutant proteins were higher than the CO ones of the corresponding proteins (Table 1). Thus, the preferential binding of O_2 over CO by the mutant protein could be achieved through a change in ρ_{Fe}. On the other hand, the O_2 affinities of L29F(7-PF) and L29F(2,8-DPF) were considerably lower than that of L29F(Proto) due to the dramatic increases in $k_{off}(O_2)$ upon CF_3 substitution(s) (Table 1). The O_2 affinity of the mutant proteins was lowered by a factor of ~1/5 on the substitution of one CF_3 group, as demonstrated for the L29F(Meso)/L29F(7-PF) system, that is, the $K(O_2)$ values of L29F(Meso) and L29F(7-PF) were 27 ± 8 μM^{-1} and 5.8 ± 2 μM^{-1}, respectively, and then by a factor of ~1/18 on the substitution of two CF_3 ones, as revealed on analysis of the L29F(3,8-DMD)/L29F(2,8-DPF) system; that is, the $K(O_2)$ values of L29F(3,8-DMD) and L29F(2,8-DPF) were 31 ± 9 μM^{-1} and 1.7 ± 0.5 μM^{-1}, respectively (Table 1). In contrast to the O_2 binding, the CO affinity of the mutant proteins was essentially independent of the CF_3 substitutions.

Vibrational Frequencies of Fe-Bound O_2 and CO of the L29F Mutant Proteins. Resonance Raman spectra of the O_2 forms of the L29F mutant proteins possessing various heme cofactors were obtained to determine the vibrational frequencies of the Fe−O stretching (ν_{FeO}) of Fe-bound O_2 in the proteins (Figure 3, Table 2, and Figures SI5−SI9 in the Supporting Information). The ν_{FeO} of 569 cm^{-1} determined for L29F(Proto) was essentially identical to that previously reported by Hirota et al.,[37] that is, 568 cm^{-1}. The ν_{FeO} value of the native protein was decreased by 1−3 cm^{-1} relative to those of the L29F mutant proteins possessing identical heme cofactors.

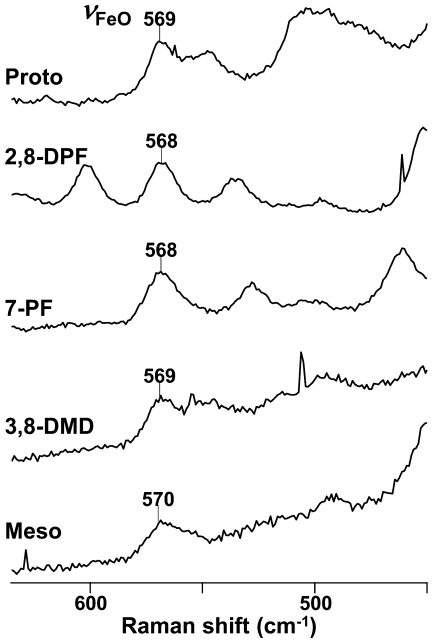

Figure 3. Visible resonance Raman spectra of O_2 forms of the L29F mutant proteins possessing the indicated heme cofactors, at pH 7.40 and 25 °C. The positions of the ν_{FeO} bands of the proteins were determined through fitting with Voigt profiles[32] (see Figures SI5−SI9 in the Supporting Information).

The ν_{CO} value, together with the Fe−C stretching (ν_{FeC}) and Fe−C−O bending frequencies (δ_{FeCO}) (Figure 4 and Figures SI10−SI16 in the Supporting Information), for the CO forms of the L29F mutant proteins possessing various heme cofactors were determined by resonance Raman spectroscopy (Table 2). The ν_{CO}, ν_{FeC}, and δ_{FeCO} of L29F(Proto) were determined to be 1933, 524, and 581 cm^{-1}, respectively, these values being essentially identical to the corresponding ones previously reported by Anderton et al.[38] The ν_{CO} values of the L29F mutant proteins were smaller by ~7−12 cm^{-1} relative to those of the native Mbs possessing identical heme cofactors, whereas these two protein systems exhibited similar ν_{CO} spans, that is, the difference between the highest and lowest ν_{CO} values within a given protein system, the values being 16 and 12 cm^{-1} for the L29F mutant protein and native Mb systems, respectively. Comparison of the ν_{CO} values of the mutant proteins yielded a difference of 8 cm^{-1} for the L29F(Meso)/L29F(7-PF) system, that is, 1928 and 1936 cm^{-1} for L29F(Meso) and L29F(7-PF), respectively, which is half the value, 16 cm^{-1}, for the L29F(3,8-DMD)/L29F(2,8-DPF) one, that is, 1928 and 1944 cm^{-1} for L29F(3,8-DMD) and L29F(2,8-DPF), respectively (Table 2). These results demonstrated the additive effect of the heme π-system perturbation on ν_{CO} for the L29F mutant proteins.

Incidently, the L29F mutant proteins exhibited a single ν_{CO} band, as shown in Figure 4, which is in contrast to the case of the native Mbs that exhibited a ν_{CO} band composed of multiple components,[19] possibly due to the presence of multiple conformational states of the Fe−CO fragment. Consequently, the Fe−CO fragment of the L29F mutant protein appeared to be forced to adapt a unique conformation through the interaction with Phe29, in addition to His64. The δ_{FeCO} values of the L29F mutant proteins were greater by ~4−7 cm^{-1} relative to those of the native Mbs possessing identical heme cofactors, possibly reflecting such a conformational constraint of the Fe−CO fragment of the mutant protein. The intensity of the δ_{FeCO} mode relative to that of the ν_{CO} mode is higher for L29F (Proto) compared with those of other species, which reproduced the reported spectra.[36]

The ν_{FeC} values of the L29F mutant proteins were greater by ~6−14 cm^{-1} relative to those of the native Mbs possessing identical heme cofactors (Table 2), and the strengthening of the Fe−C bond by the mutation, as reflected in the ν_{FeC} changes, could be in part responsible for the smaller $k_{off}(CO)$ values of the mutant proteins compared with those of the native Mbs, as shown in Table 1. Furthermore, as in the cases of various hemoproteins,[39] an inverse correlation between ν_{CO} and ν_{FeC}, expected from an admixture of the two alternative canonical forms of the Fe-CO fragment illustrated in Scheme 2,[39,40] has also been observed for the L29F mutant proteins. In addition, comparison of the ν_{FeC} values of the L29F mutant proteins revealed a decrease of 3 cm^{-1} on the substitution of one CF_3 group, as demonstrated for the L29F(Meso)/L29F(7-PF) system, that is, 526 and 523 cm^{-1} for L29F(Meso) and L29F(7-PF), respectively, and one of 10 cm^{-1} on the substitution of two CF_3 groups, as observed for the L29F(3,8-DMD)/L29F(2,8-DPF) system, that is, 528 and 518 cm^{-1} for L29F(3,8-DMD) and L29F(2,8-DPF), respectively (Table 2). Hence, in contrast to the case of ν_{CO}, an additive effect of the heme π-system perturbation was not observed for ν_{FeC}. Furthermore, the ν_{FeC} span of the L29F mutant protein system, that is, 12 cm^{-1}, was larger than that of the native Mb one, that is, 3 cm^{-1}.

DISCUSSION

Effects of Heme Cofactor Modifications on the Vibrational Frequencies of Fe-Bound O_2 and CO of the L29F Mutant Proteins. As shown in Table 2, the ν_{FeO} values

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Table 2. Vibrational Frequencies of the Fe-Bound CO and Fe-Bound O_2 of the L29F Mutant Proteins, Native Mbs, and H64L Mutant Proteins at pH 7.40 and 25 °C

	ν_{CO}[a] (cm^{-1})			ν_{FeC}[b] (cm^{-1})			$\delta_{Fe\text{-}CO}$[c] (cm^{-1})			$\nu_{Fe\text{-}CO}$[d] (cm^{-1})		
heme	L29F	Mb[e]	H64L[f]	L29F	Mb[e]	H64L[f]	L29F	Mb[e]	H64L[f]	L29F	Mb[e]	H64L[f]
Meso	1928	1939[g]	1965	526	515	489	581	576	575	570	573	573
3,8-DMD	1928	1940[g]	1964	528	514	491	583	576	575	569	571	570
7-PF	1936	1945[g]	1972	523	514	489	580	575	573	568	571	568
2,8-DPF	1944	1951[g]	1978	518	512	483	578	574	570	568	569	568
Proto	1933	1943[g]	1969	524	512	490	581	576	574	569	571	569

[a]The C−O stretching frequency of Fe-bound CO. [b]The Fe−C stretching frequency of Fe-bound CO. [c]The Fe−C−O bending frequency of Fe-bound CO. [d]The Fe−O stretching frequency of Fe-bound O_2. [e]Taken from ref 19. [f]Taken from ref 41. [g]The weighted-average ν_{CO} value taken from ref 19.

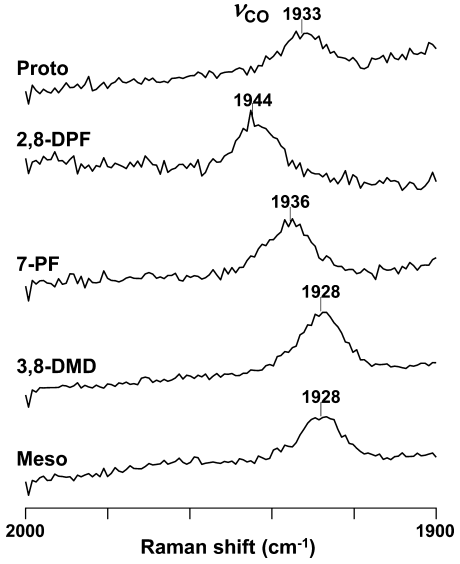
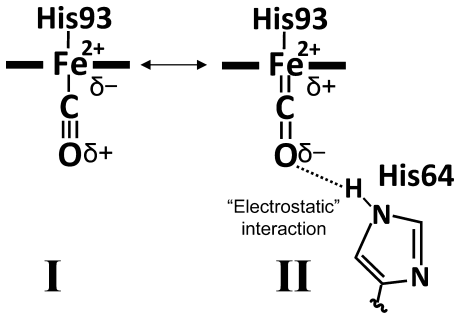


Figure 4. High-frequency regions of visible resonance Raman spectra of CO forms of the L29F mutant proteins possessing the indicated heme cofactors, at pH7.40 and 25 °C. The positions of the individual component ν_{CO} bands of the proteins were determined through fitting with Voigt profiles[32] (see Figure SI10 in the Supporting Information).

Scheme 2. Resonance between the Two Canonical Forms of the Fe^{2+}−CO Fragment of Mb[a]



[a]Represented by valence bond formalism.[38,39] The distal His64 provides a positive electrostatic potential near the O atom of Fe^{2+}-bound CO and hence contributes to stabilization of $Fe^{2+(\delta+)}$−$CO^{\delta-}$.

of the native Mbs were decreased by 1−3 cm^{-1} relative to those of the L29F mutant proteins possessing identical heme cofactors. The Fe−O_2 fragment of the O_2 form of a protein exists as an admixture of two alternative canonical forms, that is, the Fe^{2+}−O_2 and Fe^{3+}−O_2^--like species (Scheme 1).[8,22] Hence, although the resonance between the two forms is affected by the heme environment furnished by nearby amino acid residues, the Fe−O bond order is independent of the resonance,[37] leading to the low sensitivity of ν_{FeO} to replacement of amino acid residues in the heme pocket. The mutant proteins were ranked as L29F(2,8-DPF) ≈ L29F(7-PF) < L29F(3,8-DMD) ≈ L29F(Proto) < L29F(Meso), in order of increasing ν_{FeO}, although the differences are small. The weakening of the Fe−O bond in the proteins, as manifested in ν_{FeO}, however, cannot fully account for the large increase in $k_{off}(O_2)$ of the mutant proteins with decreasing ρ_{Fe}, as was reported previously for the native Mbs and H64L mutant proteins.[41]

On the other hand, the ν_{CO} value of the proteins could be reasonably interpreted in terms of the effect of the heme environment furnished by nearby amino acid residues and the heme electronic structure on the resonance in Scheme 2.[41] The L29F mutant proteins exhibited ν_{CO} values smaller by 7−12 cm^{-1} relative to those of the native Mbs possessing identical heme cofactors (Table 2). The decrease in ν_{CO} caused by a mutation could be due to the electrostatic interaction of the polar Fe(II)-bound CO with the multipole of the phenyl ring of the introduced Phe29,[20] which contributes to stabilization of Structure I over Structure II in Scheme 2. We have shown, through studies of the H64L mutant protein, where the His64 residue was replaced by Leu, that ν_{CO} is decreased by ∼20 cm^{-1} due to a positive electrostatic potential, near the Fe(II)-bound CO, provided by His64.[41] Hence, the difference of ∼30 cm^{-1} in ν_{CO} between the L29F and H64L mutant proteins possessing identical heme cofactors could be primarily interpreted in terms of the electrostatic fields provided by both Phe29 and His64. On the other hand, the difference in ν_{CO} within each of the three different protein systems could be interpreted in terms of the effect of the heme electronic structure, that is, ρ_{Fe}, on the resonance in Scheme 2. The ranking of the heme cofactors, Meso ≈ 3,8-DMD < Proto < 7-PF < 2,8-DPF, in order of increasing ν_{CO}, consistently holds for each of the protein systems. In addition, the ν_{CO} spans of the H64L mutant proteins, that is, 14 cm^{-1}, were similar to those of the L29F mutant and the native proteins, that is, 12 and 16 cm^{-1}, respectively. ν_{CO} is related to ρ_{Fe} through the resonance between the two canonical forms of the Fe^{2+}−CO fragment (Scheme 2).[39,40] The larger ρ_{Fe} is, the better the heme Fe atom can serve as a π donor to CO. The stronger the Fe^{2+}−CO bond, the greater will be the bond order of the Fe^{2+}−CO bond and the smaller will be the C−O bond order. Consequently, an increase in the ρ_{Fe} results in a decrease in ν_{CO} and vice versa. Thus, ρ_{Fe} is reflected in ν_{CO}.[41] In addition, an inverse correlation holds between ν_{FeC} and ν_{CO}, as has been demonstrated previously for various hemoproteins,[39] and a

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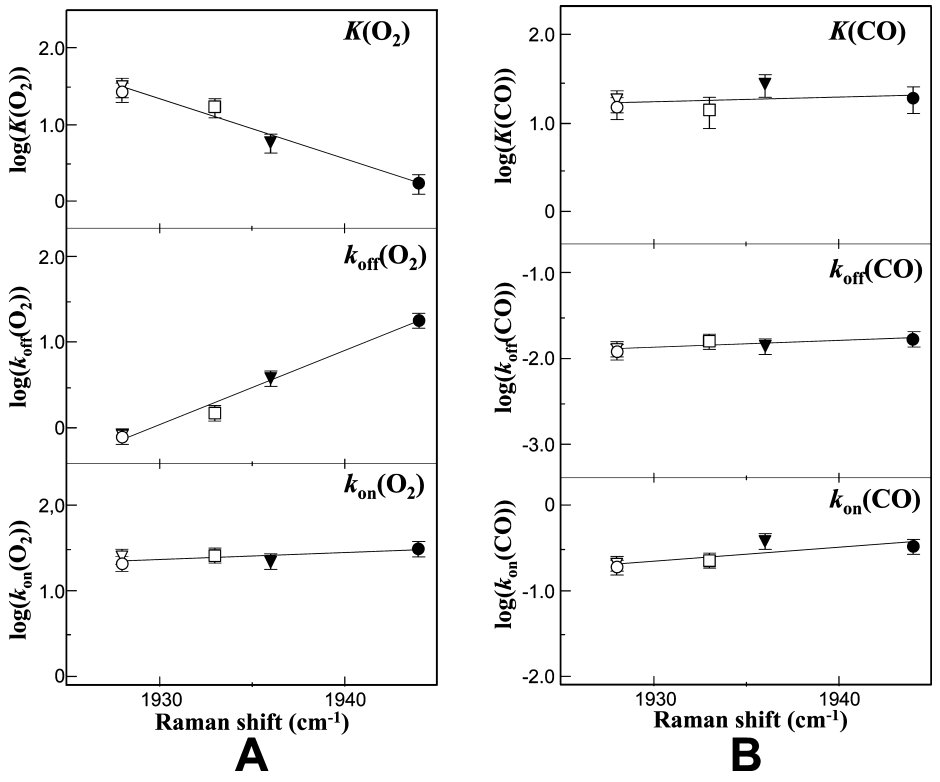

Figure 5. Plots of the quantities $\log(k_{on}(O_2))$, $\log(k_{off}(O_2))$, and $\log(K(O_2))$ against the ν_{CO} values of the L29F mutant proteins possessing Meso (▽), 3,8-DMD (○), 7-PF (▼), 2,8-DPF (●), and Proto(□) (A), and similar plots for the quantities $\log(k_{on}(CO))$, $\log(k_{off}(CO))$, and $\log(K(CO))$ (B).

similar $\nu_{FeC}-\nu_{CO}$ inverse correlation was also observed for the L29F mutant proteins.

Effects of Heme Cofactor Modifications on O_2 Versus CO Discrimination by the L29F Mutant Proteins. We showed previously that the O_2 affinity of Mb decreases (increases) due to an increase (decrease) in $k_{off}(O_2)$, with a decrease (increase) in ρ_{Fe}.[18,19] This tendency was observed for the wild-type Mbs and the H64L mutant proteins reconstituted with a variety of chemically modified heme cofactors. To determine whether the L29F mutant proteins also exhibit this tendency, we first examined plots of the quantities $\log(k_{on}(O_2))$, $\log(k_{off}(O_2))$, and $\log(K(O_2))$ against the ν_{CO} values ($\log(k_{on}(O_2))-\nu_{CO}$, $\log(k_{off}(O_2))-\nu_{CO}$, and $\log(K(O_2))-\nu_{CO}$ plots, respectively) for the L29F mutant protein system, because ν_{CO} has been shown to be a sensitive measure of the ρ_{Fe} value.[19] The plots clearly demonstrated that the O_2 affinities of the L29F mutant protein are regulated through ρ_{Fe} in a similar manner to the wild-type and H64L mutant proteins (Figure 5A). The $\log(k_{off}(O_2))-\nu_{CO}$ plots for the L29F mutant proteins could be represented by a straight line with a slope of +0.09 ($1/cm^{-1}$), and the obtained slope was similar to those of the plots for the native Mbs and H64L mutant proteins previously reported, that is, +0.1 ($1/cm^{-1}$) for the two protein systems,[41] demonstrating that the regulation of $k_{off}(O_2)$ through ρ_{Fe} is independent of the heme environment furnished by nearby amino acid residues.

Comparison of the $K(O_2)$ values of the three protein systems revealed that the $K(O_2)$ of the native protein was increased by a factor of ∼11−24 on the introduction of Phe29, through the L29F mutation, and, conversely, was decreased by a factor of ∼1/17−1/9 on the removal of His64, through the H64L one. Consequently, Phe29 in the L29F mutant protein was found to play a role as important as His64 in the native protein to enhance the O_2 affinity of the protein. Furthermore, the O_2 affinities of the L29F mutant proteins were lowered by factors of ∼1/5 and ∼1/18 on the substitution of one and two CF_3 groups, respectively. Similarly, the O_2 affinity of the native Mb was lowered by factors of ∼1/3 and ∼1/9 on the substitution of one and two CF_3 group(s), respectively, and that of the H64L mutant protein by factors of ∼1/3 and ∼1/15 on the substitution of one and two CF_3 group(s), respectively.[41] These results confirmed that the control of the O_2 affinity of the protein through ρ_{Fe} is independent of that through the heme environment furnished by nearby amino acid residues.

In contrast to O_2 binding, similar plots for CO binding, that is, $\log(k_{on}(CO))-\nu_{CO}$, $\log(k_{off}(CO))-\nu_{CO}$, and $\log(K(CO))-\nu_{CO}$ plots, for the L29F mutant protein systems indicated that the CO binding properties of the L29F mutant protein systems were not greatly affected by ρ_{Fe} (Figure 5B). The abilities of Mb to stabilize Fe(II)-bound O_2 and to discriminate against CO binding are usually evaluated on the basis of the M value, that is, $K(CO)/K(O_2)$.[6] The M value was increased by a factor of ∼5 on the substitution of one CF_3 group, as demonstrated for the L29F(Meso)/L29F(7-PF) system, and then by a factor of ∼11 on the substitution of two CF_3 groups, as observed for the L29F(3,8-DMD)/L29F(2,8-DPF) system. Plots of the quantity log M against the ν_{CO} values (log $M-\nu_{CO}$ plots) for the L29F mutant protein systems could be represented by a straight line with a slope of +0.08 ($1/cm^{-1}$). The log $M-\nu_{CO}$ plots for the native Mb and the H64L mutant protein systems could be represented by straight lines with similar slopes. These were are inevitable results, because, among the kinetic parameters for O_2 and CO binding, only $k_{off}(O_2)$ is affected by a change in ρ_{Fe}, which is

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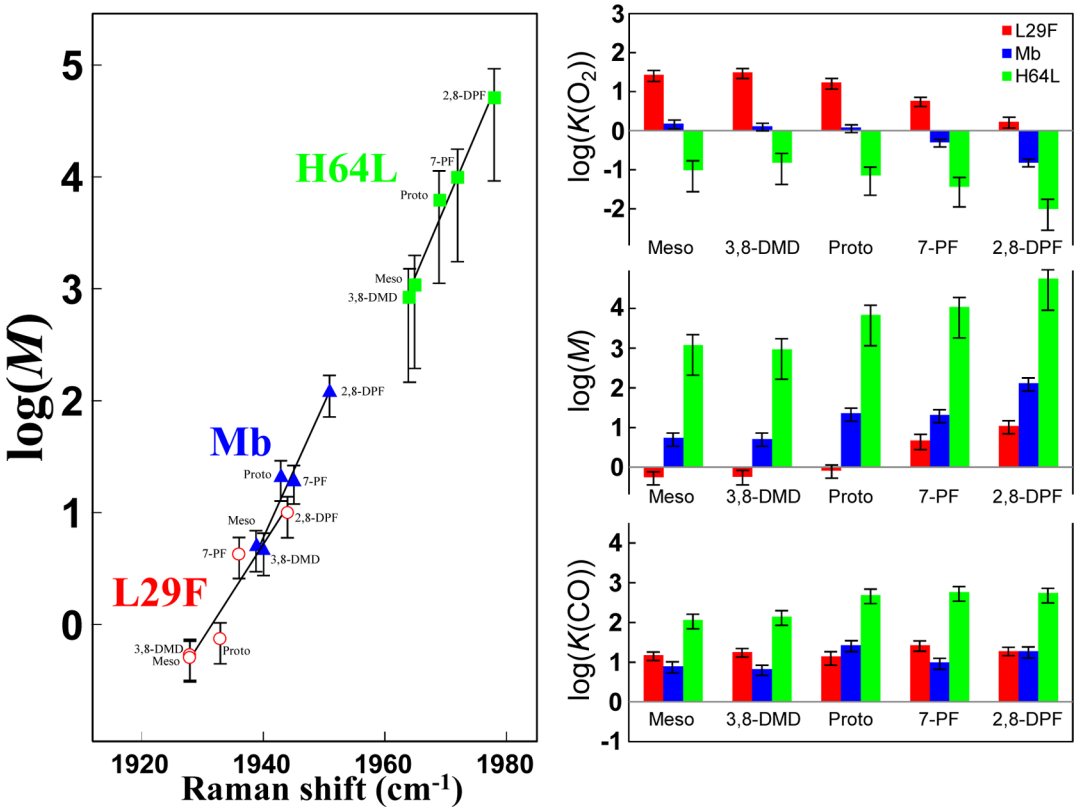

Figure 6. Plots of the quantity log(M) against the ν_{CO} values for the L29F mutant proteins (○), the native Mb (▲), and the H64L mutant proteins (■) (A). Since the native Mb exhibited a ν_{CO} band composed of multiple components, the weighted-average as to the intensity of each band was used as the ν_{CO} value for the plots.[19] Bar graphs illustrate the effects of modifications of the heme cofactors on the quantities log(K(CO)) (lower), log(M) (middle), and log($K(O_2)$) (upper) for the L29F mutant proteins (red), the native Mb (blue), and the H64L mutant proteins (green) (B).

sensitively reflected in ν_{CO},[41] in addition to the heme environment furnished by nearby amino acid residues, as described above. Furthermore, the log M–ν_{CO} plots in Figure 6A demonstrate that the M values of the proteins could be regulated over a range of ~10^5 through combined use of the amino acid replacements and the chemical modification of heme cofactors.

The three different protein systems could be ranked as L29F < native Mb < H64L, in order of increasing M value. L29F(Proto), Mb(Proto),[18] and H64L(Proto)[41] exhibited M values of 0.82 ± 0.3, 23 ± 9, and 6900 ± 5700, respectively (Table 1), and hence the M value of Mb(Proto) was increased by a factor of ~300 due to the removal of His64 through the H64L mutation, and then conversely was decreased by a factor of ~1/30 due to the introduction of Phe29 through the L29F mutation, indicating that not only His64 but also Phe29 contributes significantly to the preferential binding of O_2 over CO by the protein. Comparison of the M values of the L29F mutant proteins indicated that the substitution of one and two CF_3 groups increased the M value by factors of ~8 and ~20, respectively. The effects of the substitution of CF_3 group(s) on the M values of the L29F mutant proteins were almost the same as those of the native protein, that is, the M value of the native protein was increased by factors of ~4 and ~25 on the substitution of one and two CF_3 group(s), respectively,[18,19] again confirming that the electronic tuning of the intrinsic heme Fe reactivity through ρ_{Fe} and the heme environment furnished by nearby amino acid residues contribute independently to control of Mb function.

O_2 versus CO Discrimination by Oxygen Binding Hemoproteins. Two types of Hb or Mb have been reported to exhibit M values of less than unity. One is Hbs derived from parasitic nematodes *Pseudoterranova decipiens*[42] and *Ascaris suum*[42] and trematodes *Paramphistomum epiclitum*,[43] *Gastrothylax crumenifer*,[44] and *Explanatum explanatum*.[44] The other is Mb reconstituted with an iron-porphycene complex.[27] Monomeric *Paramphistomum epiclitum* Hb (PeHb) has been subjected to systematic mutation studies to elucidate the molecular mechanism responsible for control of its ligand-binding properties.[43] PeHb possesses two tyrosine (Tyr) residues at the B10 and E7 positions (the protein moiety of the Hb subunit or Mb generally consists of eight helices, labeled A through H, and B10 represents the 10th residue in the B helix, and so forth), and it exhibits an M value of 0.19.[43] PeHb exhibited an unusually small $k_{off}(O_2)$, that is, 0.07 s^{-1}, whereas $k_{on}(O_2)$, k_{on}(CO), and k_{off}(CO) of the protein were similar to the corresponding ones of the Mb mutant proteins possessing an apolar residue at the E7 position such as H64L(Proto).[45] The remarkably small $k_{off}(O_2)$ value of PeHb has been proposed to be due to the stabilization of Fe(II)-bound O_2 through hydrogen bonding with the two Tyr residues at the B10 and E7 positions, in addition to other nearby residues.[44] On the other hand, its relatively large $k_{on}(O_2)$ and k_{on}(CO) values are likely inherited from a protein folding motif characteristic of O_2 binding hemoproteins, the so-called globin fold, with a low kinetic barrier for ligand entry,[46] because these values are thought to be primarily determined by the heme pocket polarity and the accessibility of an exogenous ligand to the heme pocket.[43] In addition, k_{off}(CO) of PeHb reflects the

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intrinsically great stability of Fe(II)-bound CO in O_2 binding hemoproteins. Thus, the preferential binding of O_2 over CO by PeHb is achieved through the remarkably high O_2 affinity due to the considerable reduction of $k_{off}(O_2)$. In a similar way, the preferential binding of O_2 over CO by Mb reconstituted with an iron-porphycene complex is achieved through a marked reduction of $k_{off}(O_2)$.[28] Similarly, as shown in Figure 6B, electronic control of the O_2 versus CO discrimination by Mb described in this study relies on the effect of a change in ρ_{Fe} solely on $k_{off}(O_2)$.

CONCLUSION

The O_2 versus CO ligand-binding preference of the L29F mutant proteins of myoglobin has been shown to be regulated through a change in the electron density of the heme Fe atom. The study confirmed that the effects of a change in the electron density of the heme Fe atom and the heme environment furnished by nearby amino acid residues on the functional properties of the protein are independent of each other, and the ratio between the equilibrium constants for CO and O_2 binding by the protein can be regulated over a range of $\sim 10^5$ through combined use of the chemical modification of heme cofactors and the replacements of amino acid residues in the distal heme pocket. These findings provide useful insights for designing functional properties of O_2 binding hemoproteins.

ASSOCIATED CONTENT

Supporting Information

Plots showing the evolution of absorbance changes at indicated wavelengths, pseudo-first-order rate constants, and Raman spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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