Conversion of an Electron-Transfer Protein into an Oxygen Binding Protein: The Axial Cytochrome b_5 Mutant with an Unusually High O_2 Affinity

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Received August 7, 2000

Cytochrome b_5 (cyt b_5) is a small hemoprotein that functions as an electron mediator and possesses a six-coordinated heme ligated by histidine-39 (His39) and histidine-63 (His63) (Figure 1A). While cyt b_5 cannot bind exogenous ligands and does not exhibit catalytic activities, several groups have converted the function of cyt b_5 by replacing one of the axial histidines.[1] For example, Sligar et al. showed that the replacement of His39 for Met (H39M) renders the variant exhibiting a reversible CO binding and a peroxidase activity.[1a] However, no mutant of cyt b_5 has been reported to exhibit a reversible O_2 binding, because interactions that stabilize the oxygen binding are difficult to design. In this article, we report newly constructed mutants of rat hepatic cyt b_5 exhibiting reversible oxygen binding with high affinities.

We initiated our project by creating a ligand binding site at the His39 side of the heme, because our previous work demonstrated the importance of the heme binding motif including His63 for the stable heme association of cyt b_5.[2] The H39L mutant in which His39 is replaced with Leu exhibited spectral characteristics that are similar to those of myoglobin (Mb) (Figure 2), and demonstrated a reversible binding of O_2. On the other hand, the H63L mutant cannot form a dioxygen adduct due to its rapid autoxidation. We determined the O_2 association rate constant for H39L by the laser photolysis measurement[3] and the O_2 dissociation rate constant by the replacement reaction[4] of bound O_2 with CO. The resultant O_2 association (36 μM^{-1} s^{-1}) and dissociation (1.4 s^{-1}) rate constants for H39L at 10 °C were about 4-fold faster and 4-fold slower than those for Mb (Table 1), respectively. The faster association rate is probably due to the less crowded distal structure of H39L. The slower dissociation rate indicates that the Fe−O_2 bond for H39L is more stable than that for Mb. Because the mutant does not possess a distal histidine that plays a key role in stabilizing the Fe−O_2 complex by a hydrogen bonding in Mb,[5] we investigated the stabilization mechanism for the Fe−O_2 bond of the mutant.

We examined the NMR structure of wild-type cyt b_5[6] to identify possible stabilizing contacts for the Fe−O_2 bond of H39L. We found two candidates for the hydrogen-bonding partner of the coordinated O_2; an amide proton of Gly41 and a hydroxyl proton of Tyr74 that are located at 5.3 and 10 Å from the heme iron, respectively (Figure 1A). While Tyr74 is too separated from the bound O_2, we considered that certain structural changes might bring Tyr74 in the vicinity of the bound O_2. In addition, we found that a positive edge of Phe35 which is located at 4.6 Å from the heme iron might stabilize the bound O_2 by the electrostatic interaction[7] (Figure 1A). We will examine these three possibilities below.

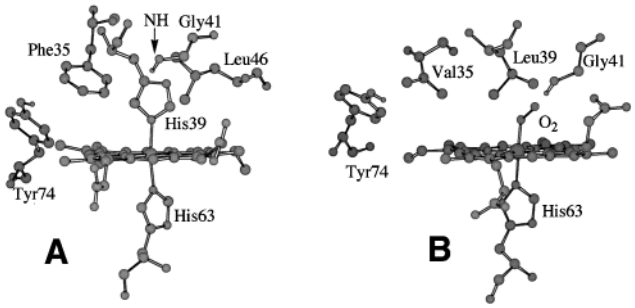

Figure 1. (A) The heme environmental structure of rat cyt b_5 determined by NMR.[6] (B) The putative structure of the O_2-bound form for F35V/H39L calculated using Discover Release 97.0 (MSI, Inc). During the molecular modeling, we constricted the movement of Leu46 within 0.5 Å from the wild-type location.

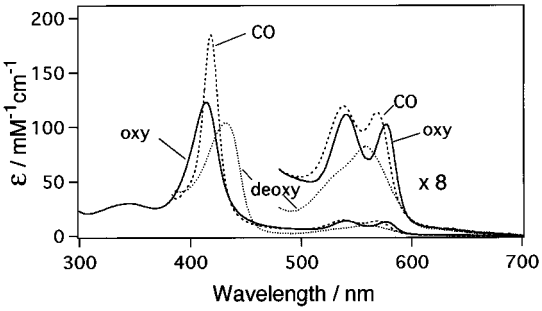

Figure 2. Absorption spectra of the oxy (solid line), CO (dashed line) and deoxy forms (dotted line) for H39L. The peaks are observed at 414.5, 540.5, and 576.5 nm for the oxy form, 419.5, 537.5, and 568 nm for the CO form, 430.5 and 558 nm for the deoxy form. The absorption spectra of F35V/H39L are identical with those of H39L, except for the Soret peak of the CO form (417.5 nm for F35V/H39L).

Table 1. Ligand Binding Parameters and the Frequencies of C═O Stretching Mode of the Cyt b_5 Mutants; the Corresponding Values for Mb (human) and Plant Hbs Are Also Presented for Comparison

	$k_{on(O_2)}$ (μM^{-1} s^{-1})	$k_{off(O_2)}$ (s^{-1})	$K_{(O_2)}$ (μM^{-1})	K_{CO}/K_{O_2}	C═O mode cm^{-1} (%)
Mb[a]	8.4	5.6	1.6	22	1945[f]
H39L[a]	36	1.4	26	48	1933(15), 1970(85)[f]
Y74F/H39L[a]	27	0.37	73	71	1931(15), 1970(85)[f]
F35V/H39L[a]	9.7	0.0073	1300	2.0	1929(90), 1969(10)[f]
F35V/H39L[b]	26	0.078	330		
BarleyHb[c]	9.5	0.027	350	1.5	1924(90), 1960(10)[g]
*Arabidopsis*Hb[d]	75	0.12	630	2.0	
RiceHb[e]	68	0.038	1800	4.0	

[a] This study. Obtained at 10 °C and pH 8.0. [b] This study. Obtained at 20 °C and pH 8.0. [c] Duff et al.[14a] Obtained at 20 °C and pH 7.5. [d] Trevaskis et al.[14b] Obtained at 20 °C and pH 7.0. [e] Arredondo-Peter et al.[14c] [f] This study. Obtained at room temperature and pH 8.0. [g] Das et al.[15a]

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(5) As summarized in the several reviews [(a) Springer, B. A.; Sligar, S. P.; Olson, J. S.; Phillips, G. N. Jr. *Chem. Rev.* **1994**, *94*, 699−714. (b) Olson, J. S.; Phillips, G. N. Jr. *J. Biol. Inor. Chem.* **1997**, *2*, 544−552.], many site-directed mutagenesis and spectroscopic studies have shown that Mb stabilizes the partial negative charge of the polar Fe−O_2 complex by the hydrogen bonding.

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10.1021/ja002914r CCC: $19.00
Published on Web 11/03/2000

We first constructed two double mutants, Y74F/H39L and F35V/H39L, to investigate the contributions from Tyr74 and Phe35, respectively. The observed O_2 association (27 μM^{-1} s^{-1}) and dissociation (0.37 s^{-1}) rate constants for Y74F/H39L were similar to those for H39L (Table 1), demonstrating that the hydrogen bond between the coordinated O_2 and the hydroxyl group of Tyr74 is absent in H39L. On the other hand, the ligand binding property of F35V/H39L is astonishing because of an unusually slow dissociation rate of the bound O_2 (0.0073 s^{-1} at 10 °C). The affinity for O_2 in F35V/H39L (1300 μM^{-1}) is 50-fold higher than that in H39L (Table 1). Furthermore, the ferrous heme-iron in the O_2 bound form of F35V/H39L has a much slower autoxidation rate constant (0.38 h^{-1} at 20 °C) than that of H39L (6.6 h^{-1} at 20 °C). These results rule out the contribution of Phe35 to the stabilization of the Fe−O_2 bond in H39L. To explain the enhanced stability in F35V/H39L, we interpret that the stabilization effect in H39L is strengthened in F35V/H39L.

The remaining candidate for the stabilization of the bound O_2 in the cyt b_5 mutants is the amide proton of Gly41. To examine the presence of the hydrogen bond, we measured the infrared (IR) spectra of the CO form of the constructed mutants (Table 1). The C═O stretching mode is sensitive to polar interactions of the bound CO with the distal pocket including the hydrogen bonding.[8] In the IR spectra of H39L and F35V/H39L, two C═O stretching modes are observed at ∼1930 and ∼1970 cm^{-1}. The mode at ∼1930 cm^{-1} for both mutants exhibits a downshift by 1 cm^{-1} in the D_2O buffer, which provides a direct evidence for the interaction of exchangeable proton(s) with the bound CO.[9] The proton(s) is likely donated from the amide of Gly41 or a sequestered water molecule[10] trapped by Gly41. It is noteworthy that the population of the mode at ∼1930 cm^{-1} is much larger for H35V/H39L (90%) than that for H39L (15%). Phillips et al. correlated the C═O stretching frequencies of the CO bound form of Mbs with the extent of the electrostatic stabilization of the bound O_2,[8c] and concluded that the frequencies of 1930 and 1970 cm^{-1} correspond to very strong and little stabilizations of the Fe−O_2 bond, respectively. Therefore, the difference in the O_2 dissociation rates of the mutants is consistent with the difference in the population of the mode at 1930 cm^{-1}.

The proposed hydrogen-bonding stabilization of the Fe−O_2 bond using Gly41 is based on the assumption that the distal pocket structures of the mutants are similar to that of wild-type cyt b_5. To delineate the structural changes induced in the heme pocket of the mutants, we carried out the preliminary ^{1}H NMR studies of their CO adduct. While we failed to assign the peaks from Gly41 for the mutants,[11] several ring-current shifted peaks were assigned by using COSY and NOESY spectra (data not shown). We used the peaks from Leu46 located adjacent to Gly41 (Figure 1A) as the markers of the distal structure, which were observed at −0.89 ($C^{\alpha}H$), −1.00 ($C^{\alpha}H$), −1.02 ($C^{\alpha\prime}H$), and −1.14 ($C^{\alpha\prime}H$) ppm for wild-type cyt b_5, −0.35 ($C^{\alpha}H$) and −0.73 ($C^{\alpha\prime}H$) ppm for H39L and −0.73 ($C^{\alpha}H$), −0.81 ($C^{\alpha}H$), −1.12 ($C^{\alpha\prime}H$), and −1.22 ($C^{\alpha\prime}H$) ppm for F35V/H39L.[12] According to the relationship between the ring current shift and the distance from the heme iron to the proton,[13] the shifts indicate that Lue46 in H39L and F35V/H39L are located within 1 and 0.3 Å, respectively, from the wild-type location. We, therefore, conclude that there are no significant structural changes in the distal site of the mutants. As shown in the putative structure of the oxygen bound form for F35V/H39L (Figure 1B), Gly41 is likely located adjacent to the ligand binding site of the mutants and can stabilize the Fe−O_2 bond by the hydrogen bond.

It should be noted that the O_2 binding parameters for F35V/H39L are similar to those for plant hemoglobins[14] (Hbs) such as Barley, *Arabidopsis*, and Rice Hbs, which possess the highest O_2 affinities among the known hemoproteins (Table 1). Consistent with our proposal, Barley Hb exhibits the C═O stretching frequencies similar to those of F35V/H39L (Table 1), and plant Hbs are proposed to possess the strong hydrogen bond.[14,15] It is also interesting to note that a similar stabilization mechanism of the Fe−O_2 bond using the amide proton of Gly was suggested for heme−heme oxygenase complex exhibiting slow O_2 dissociation rate (0.25 s^{-1}).[10a,16]

In summary, we have successfully converted the typical electron-transfer hemoprotein, cyt b_5, into the artificial oxygen binding hemoprotein.[17] The O_2 affinity for F35V/H39L is 800-fold and 130-fold higher than those of Mb and the axial ligand mutant of cytochrome *c*,[18] respectively. The cytochrome *c* mutant has been a unique artificial hemoprotein with the O_2 binding ability. On the basis of the IR and NMR studies, we suggest the stabilization of the Fe−O_2 bond by the hydrogen bond either from the amide proton of Gly41 or a water molecule ordered by the amide proton. To approach the detailed stabilization mechanisms of the mutants, NMR and kinetic studies are currently in progress.

Acknowledgment. We are grateful to Professor S. G. Sligar (University of Illinois) for a gift of expression vector of the rat cytochrome b_5 gene. This work was supported by grants-in-aid for scientific research on priority areas "Molecular Biometallics" (08249102 to I.M.) and "Principles of Protein Architecture" (07280101) from the Ministry of Education, Science, Culture and Sports.

Supporting Information Available: IR spectra of the CO bound form of H39L and F35V/H39L obtained in 99% D_2O and H_2O (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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