Engineering a Hydrophilic Heme Catalytic Pocket into Microsomal Cytochrome b_5: Construction of Novel Metalloproteins with High Peroxidase-like Activity

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We have engineered novel hemoproteins with high catalytic activity by introducing several hydrophilic residues in the axial ligand of microsomal cytochrome b_5, an electron transfer protein. Some (H39S and H39Q) of these mutants possess quite high peroxidase-like reactivity as designed. One (H39C) of them shows very low catalytic reactivity, and this may be attributed to the unique hexa-coordination (His-Fe^{3+}-Cys) for this mutant.

Hemoprotein is an excellent natural model and starting-molecule for artificial design of metalloproteins with desired or novel catalytic reactivity. Various heme-containing proteins show an overall-structural similarity but a functional diversity. One of the most significant structural characteristics governing functional properties of hemoproteins is the micro-environment of the heme,[1] including the coordination structure of heme iron and properties of coordination sphere pocket. So, the artificial engineering of heme pocket could be a crucial strategy to generate new hemoproteins with novel reactivity.[2]

A common and essential structure characteristic of catalytic hemoproteins is a penta-coordination of their active center, the heme iron, so providing a distal pocket accessible to molecular oxygen, peroxides, and other substrates. For example, the well-characterized electron transfer hemoprotein, microsomal cytochrome b_5 (cyt b_5),[3] which has a bis-histidine (His39, His63) hexa-coordination environment, could be converted into catalytic species by replacement of the heme axial ligand with non-coordinating hydrophobic residue.[4] Rational design for the catalytic mutant proteins will encounter the consideration: how to improve their catalytic reactivity as high as possible? The engineering of a somewhat more hydrophilic heme distal pocket may be a valuable choice. Not only could this improve substrate affinity of the heme cleft, but also aid to stabilize reaction intermediates through secondary bonds.[5] To test this idea, in the present study, we have constructed several hydrophilic heme pockets of microsomal cyt b_5. Drastic high peroxidase-like reactivity was indeed found in some mutants (e.g., the H39S variant). Furthermore, a significant difference in catalytic reactivity observed in various mutants is attributable to the different heme coordination environment of these mutant proteins.

To achieve the above purpose in a simple model, we have replaced His39, one of the two heme axial ligands of cyt b_5, with suitable hydrophilic residue. Three particular axial ligand mutants of bovine liver microsomal cyt b_5: H39S, H39Q, and H39C were selected from an axial ligand gene mutant library built in our lab and to be expressed into proteins.[6] Different heme iron coordination environments were detected in these cyt b_5 variants. As expected, the H39S and H39Q mutants that hold an open hydrophilic distal pocket both show very high peroxidase-like activity. But the H39C mutant shows very low catalytic activity. This may be due to the unique hexa-coordination (His-Cys) for its ferric heme.

The electronic absorption spectra of the oxidized H39S and H39Q mutant all show Soret band at 405 nm and the high-spin (HS) markers around 630 nm (Figure 1). These spectra are very similar to that of the wild type aquo-metmyoglobin [λ_{max} (nm): 408, 502, 630] which has the HS heme with proximal histidine and distal water as the axial ligands.[7] This suggests the HS ferric hemes in these mutants are all coordinated by His63 in the proximal side and by water in the distal pocket. The ferrous H39S and H39Q mutants, however, appear to be mainly in the low-spin (LS) states, as indicated by the resolved α and β bands observed in their electronic spectra (Figure 1). So, Ser39 (or Gln39) as well as His63 are considered to be coordinated to the ferrous heme under this condition. This kind of absorption spectra and heme coordination environments similar to those of H39S and H39Q mutants, were also reported previously.[4] The electronic spectrum of the oxidized H39C mutant [λ_{max} (nm): 359, 423, 542, 575] (Figure 2) is strikingly similar to those of the ferric CO-sensing CooA protein [λ_{max} (nm): 362, 424, 540, 574][8] and ferric cyt *P450*-imidazole complex [λ_{max} (nm): 360, 425, 541, 578],[9] which were reported to contain the LS heme coordinated by cysteine and histidine (or exogenous imidazole) ligands. This indicates that the LS character and the Cys39-His63 heme axial ligation in the ferric H39C mutant.[10] The ferrous H39C mutant (Figure 2) shows the absorption spectrum [λ_{max} (nm): 429, 559] close to those of the reduced forms of human myoglobin [λ_{max} (nm): 433, 558][11a] and horse heart myoglobin [λ_{max} (nm): 435, 560],[11b] which were known to have the HS penta-coordinated ferrous heme iron. These results indicate the heme of ferrous H39C mutant is in a HS state and only coordinated by the proximal His63 under this

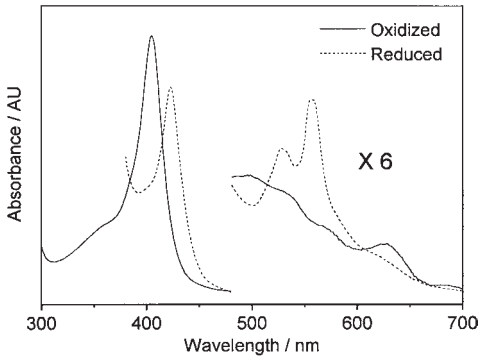

Figure 1. Electronic spectra of the H39S mutant of microsomal cytochrome b_5. The H39Q mutant has analogue absorption spectra of H39S in both oxidation states.

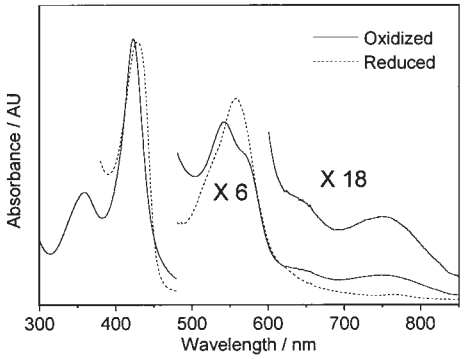

Figure 2. Electronic spectra of the H39C mutant of microsomal cytochrome b_5.

experimental condition. The coordination of the H39C mutant is unique in mimicking heme coordination (His-Cys) of the CO-sensing CooA signal transduction hemoprotein up to now.

Peroxidase-like activities of His39 mutants of cyt b_5 were examined by using a characteristic oxidation reaction of o-methoxyphenol to its tetramer.[12,13] The determined initial rates of the product formation are summarized in Table 1. The H39S and H39Q mutants show much higher peroxidase activities than previously reported.[4] Probably, this is due to readily openness of the heme sixth coordination position, the more hydrophilic distal pocket and the possible hydrogen bonds formed between the Ser39 (or Gln39) and the bound H_2O_2. In the case of the H39C mutant, however, its kinetic reaction trace shows an obvious sigmoid type process, indicating the existence of a slow phase followed by a fast phase. And even at the fast phase, the reaction rate is still much lower than the other mutants. No doubt, this is due to the unique hexa-coordination for the ferric heme of the mutant. The substantially low catalytic activity of the H39C mutant in the slow phase implies that the coordination of Cys39 prevents the binding of H_2O_2 to heme as the situation in the wild type cyt b_5. In the fast phase, exogenous ligands compete over Cys39 for the heme sixth coordination, but Cys39 still severely inhibits the catalytic reaction.

Table 1. Initial rates of tetraguaiacol formation of cytochrome b_5 variants[a]

Enzyme	Initial rate /μM min^{-1}	Catalytic activity[b]	Relative activity
H39S	9.441	1.888	193
H39Q	5.548	1.110	113
H39C (slow phase[c])	0.193	0.039	4
H39C (fast phase[d])	0.573	0.115	12
Hemin alone (5 μM)	0.532	0.106	11
WT cyt b_5	0.049	0.010	1

[a]Experimental conditions see context. [b]μM tetraguaiacol product formation per minute per μM enzyme. [c]From 0 to 120 s after start of the reaction. [d]From 120 to 360 s after start of the reaction.

In summary, we have successfully engineered a particular hydrophilic heme distal pocket into microsomal cyt b_5 for the first time, and as expected, it is truly very helpful to improve the catalytic activities of the artificial hemoproteins. Study presented here perhaps throw light on one aspect of the protein engineering of artificial heme-based bio-catalysts, further steps would include appropriate structural modification in order to stabilize the heme active motif.[14]

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References and Notes

1 A. L. Raphael and H. B. Gray, *J. Am. Chem. Soc.*, **113**, 1038 (1991); see also references therein.
2 S.-I. Ozaki, T. Matsui, M. P. Roach, and Y. Watanabe, *Coord. Chem. Rev.*, **198**, 39 (2000).
3 a) R. C. E. Durley and F. S. Mathews, *Acta Crystallogr., Sect. D*, **52**, 65 (1996). b) J. Wu, Z.-X. Xia, Y.-H. Wang, W.-H. Wang, L.-L. Xue, Y. Xie, and Z.-X. Huang, *Proteins: Struct., Funct., Genet.*, **40**, 249 (2000). c) Y.-L. Sun, Y.-H. Wang, M.-M. Yan, B.-Y. Sun, Y. Xie, Z.-X. Huang, S.-K. Jiang, and H.-M. Wu, *J. Mol. Biol.*, **285**, 347 (1999). d) L.-L. Xue, Y.-H. Wang, Y. Xie, P. Yao, W.-H. Wang, W. Qian, and Z.-X. Huang, *Biochemistry*, **38**, 11961 (1999).
4 S. G. Sligar, K. D. Egeberg, J. T. Sage, D. Morikis, and P. M. Champion, *J. Am. Chem. Soc.*, **109**, 7896 (1987).
5 T. Matsui, S. Ozaki, and Y. Watanabe, *J. Am. Chem. Soc.*, **121**, 9952 (1999).
6 The purified cyt b_5 variants were confirmed by the mass spectroscopy on a Bruker Esquire 3000 Electrospray Mass Spectrometer. The measured molecular weights are: 10634.96 ± 0.5 (wild type); 10585.87 ± 0.5 (H39S); 10625.99 ± 0.5 (H39Q); and 10600.95 ± 0.5 (H39C), that correspond well to the values calculated from amino acid compositions of apo-proteins (10634.58, 10584.51, 10625.57, and 10600.47 Da, respectively).
7 M. Antonini and E. Brunori, in "Hemoglobin and myoglobin in their reactions with ligands," ed. by A. Neuberger and E. L. Tatum, North-Holland Publishers, Amsterdam (1971).
8 M. F. Reynolds, D. Shelver, R. L. Kerby, R. B. Parks, G. P. Roberts, and J. N. Burstyn, *J. Am. Chem. Soc.*, **120**, 9080 (1998).
9 J. H. Dawson, L. A. Andersson, and M. Sono, *J. Biol. Chem.*, **257**, 3606 (1982).
10 In addition, Cys39, the unique sulfur-containing residue of the H39C mutant, is coordinated to the ferric heme, which is confirmed by the sulfur to Fe(III) charge-transfer bands resolved at lower energy region (650 and 750 nm).
11 a) S.-I. Adachi, S. Nagano, Y. Watanabe, K. Ishimori, and I. Morishima, *Biochem. Biophys. Res. Commun.*, **180**, 138 (1991). b) E. Lloyd, D. P. Hildebrand, K. M. Tu, and A. G. Mauk, *J. Am. Chem. Soc.*, **117**, 6434 (1995).
12 Peroxidase-like activities were conducted with 5 μM of cyt b_5 variants (all in their natural oxidized states) in 2.5 mL of 100 mM sodium phosphate buffer at pH 8.0, containing 10 mM of o-methoxyphenol as substrate, and the reaction temperature was $20 \pm 0.2\,^{\circ}$C. The reaction was initiated by the addition of hydrogen peroxidase to the starting mixtures (at a final concentration of 1.0 mM) and followed by monitoring the absorbance change of the product tetraguaiacol at 470 nm ($\varepsilon_{470} = 26.6\,\mathrm{mM^{-1}\,cm^{-1}}$).
13 a) A. Fujita, H. Senzu, T. Kunitake, and I. Hamachi, *Chem. Lett.*, **1994**, 1219. b) S. Sakamoto, I. Obataya, A. Ueno, and H. Mihara, *J. Chem. Soc., Perkin Trans.* 2, **1999**, 2059.
14 T. Uno, A. Yukinari, Y. Moriyama, Y. Ishikawa, Y. Tomisugi, J. A. Brannigan, and A. J. Wilkinson, *J. Am. Chem. Soc.*, **123**, 512 (2001).