Our work demonstrates the pivotal role played by the spacer in determining the effectiveness of complexation by molecular tweezers. In this regard, optimum binding affinities will be achieved if a suitably sized spacer is both rigid *and* capable of enforcing a syn-cofacial orientation of the complexing chromophores. Further strengthening of the complexation in our system can be expected from improved electron donor-acceptor (EDA) interactions[16b] and involvement of the hydrophobic effect.

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Alteration of Heme Axial Ligands by Site-Directed Mutagenesis: A Cytochrome Becomes a Catalytic Demethylase

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Cytochrome b_5 is a 17000 dalton protein that serves an electron transport role in the hepatic endoplasmic reticulum, coupling to the fatty acid desaturase complex[1] and cytochrome P-450.[2] A similar cytochrome b_5, lacking a 35 amino acid membrane anchor domain, acts as a soluble reductant of hemoglobin in erythrocytes.[3,4] The redox active center of cytochrome b_5 is iron-protoporphyrin IX ligated to histidine-39 and histidine-63 of the polypeptide chain, and the complete three-dimensional structure of the water-soluble core domain is known from X-ray[5] and NMR[6] investigations.

An important goal of bioinorganic chemistry is elucidating the role of metal ligands in determining the chemical and spectroscopic properties of metalloprotein prosthetic groups. Recently, we reported the total synthesis of a gene coding for rat liver cytochrome b_5 and the high-level expression of this gene in a bacterial environment.[7] With the ability to genetically engineer cytochrome b_5 via in vitro recombinant DNA technology, we now report the replacement of the axial histidine residue of this protein at position 39 with a methionine residue in order to mimic the coordination geometry of cytochromes *c* and b_{562}.[8] The purified methionine-39

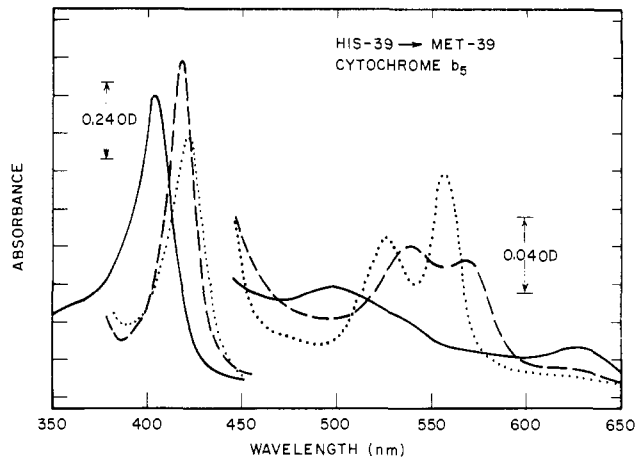

Figure 1. Ferric (solid), ferrous (dotted), and ferrous-carbon monoxide (dashed) optical spectra of H39M-b_5. The low-spin character of the ferrous protein is indicated by the resolved visible bands, and the high-spin nature of the ferric state by the 625-nm charge-transfer transition.

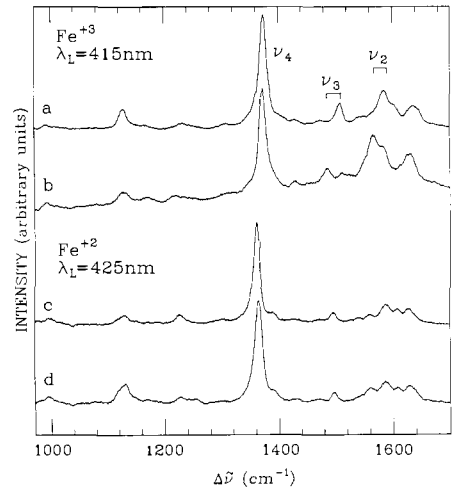

Figure 2. Resonance Raman spectra of native (a,c) and mutant (b,d) cytochrome b_5. The laser excitation wavelengths for oxidized (a,b) and reduced (c,d) proteins are indicated. The spin state and coordination state can be assigned on the basis of the core size marker band ν_3. The oxidation state is assigned based on the marker frequency ν_4 (see Table I).

mutation of cytochrome b_5, hereafter termed H39M, has the optical spectra shown in Figure 1. Electron spin resonance spectra of H39M showed primarily high-spin heme with slight rhombic distortion (g = 6.25, 5.73, 1.99) and a small low-spin component comprising roughly 5% of the total signal with g values of 2.87, 2.28, 1.60. As demonstrated by Figure 1, ferrous H39M-cytochrome b_5 readily binds carbon monoxide to form a stable $Fe^{2+}CO$ state. In Figure 2 we display the Raman spectra of native b_5 and the H39M mutant. The ferric mutant is assigned as six-coordinate high spin; however, a small amount of low spin is present as suggested by the weak band at 1510 cm^{-1} in agreement with the EPR data. At present it is not known whether the methionine-39

(1) Oshimo, N.; Sato, R. *J. Biochem.* (*Tokyo*) **1971**, *69*, 169-180. Strittmatter, P.; Spatz, L.; Corcoran, D.; Rogers, M. J.; Redline, R. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4565-4569. Shimakata, T.; Mihara, K.; Sato, R. *J. Biochem.* (*Tokyo*) **1972**, *72*, 1163-1174.

(2) Cohen, B.; Estabrook, R. W.; *Arch. Biochem. Biophys.* **1971**, *143*, 54-65. Hildebrant, S. G.; Estabrook, R. W. *Arch. Biochem. Biophys.* **1971**, *143*, 66-79. Morgan, E. T.; Coon, M. J. *Drug Metab. Dispos.* **1984**, *12*, 358-364. Tamburini, P. P.; White, R. E.; Schenkman, J. B. *J. Biol. Chem.* **1985**, *260*, 4007-4015.

(3) Hultquist, D.; Passon, P. *Nature* (*London*) *New Biol.* **1971**, *229*, 252-254. Hultquist, D.; Sannes, L.; Schafer, D. *Prog. Clin. Biol. Res.* **1981**, *55*, 291.

(4) Hegesh, E.; Hegesh, J.; Kaftory, A. *New England Journal of Medicine* **1986**, *314*, 757-761.

(5) Mathews, F. S.; Argos, P.; Levine, M. *Cold Spring Harbor Symp. Quant. Biol.* **1972**, *36*, 387-393.

(6) Keller, R. M.; Wüthrich, K. *Biochem. Biophys. Acta* **1980**, *621*, 204-217.

(7) Beck von Bodman, S.; Schuler, M.; Jollie, D.; Sligar, S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9443-9447.

(8) Methodologies for substitution of the histidine axial ligands are described in ref 7. In order to generate His-39 mutants, the wild-type b_5 synthetic gene was cut with the restriction enzymes *SmaI* and *SalI*, and the large fragment was isolated by gel electrophoresis. The two wild-type oligonucleotides, corresponding to positions 98-125 in the sense and 102-136 in the anti-sense strand, were mixed with equal molar amounts of two new oligonucleotides where the histidine codon CAC was replaced by CTG (Leu), ATG (Met), and GTG (Val), and the adjacent proline codon (CCC) was replaced by CCG. This latter replacement deletes the *SmaI* site and provides for easy screening of recombinants. The four oligonucleotides were mixed with *SmaI*-*SalI* cut DNA at a 3:1 molar ratio, heated to 90 °C, cooled slowly, ligated with T4 DNA ligase, and transformed into *E. coli* TB-1 (BRL Incorporated). Recombinant colonies were screened by colony hybridization and were verified by DNA sequencing.

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Table I. Raman Marker Bands of Cytochrome-b_5 and H39M

system	oxidtn	ν_4 (cm^{-1})	spin	coordntn	ν_3 (cm^{-1})
			5/2	6	1478–1488
heme[a]	Fe^{3+}	1368–1377	5/2	5	1491–1500
			1/2	6	1502–1507
heme[a]	Fe^{2+}	1344–1364	2	5	1471–1474
			0	6	1490–1498
Cyt-b_5	Fe^{3+}	1375	1/2	6	1510
H39M	Fe^{3+}	1373	5/2	6	1486
Cyt-B_5	Fe^{2+}	1361	0	6	1496
H39M	Fe^{2+}	1364	0	6	1497

[a] Range of values for ν_3 and ν_4 observed in heme proteins and model compounds having specified oxidation state, spin state, and coordination number.

or a water molecule is the heme axial ligand in the ferric state. The formation of the ferrous–carbon monoxide adduct is confirmed by the shift of ν_4 into the region associated with ferric species. The presence of an iron–carbon monoxide stretch at 500 cm^{-1} (data not shown) indicates that histidine rather than methionine is the axial ligand. (Sulfur ligation usually results in ~20 cm^{-1} downshift of this mode.) No stable O_2 adduct is generated; rather, in the presence of oxygen, the ferric state is immediately regenerated at room temperatures, indicating facile autoxidation of the metal center.

The effect of axial ligands on the redox potential of metal centers has been a subject of widespread interest.[9] In general, the presence of an electron rich oxygen of water (or sulfur of methionine) in the mutant cytochrome b_5 versus the histidine of the wild-type b_5 would tend to stabilize the increased positive charge of the ferric state and hence lower the observed redox potential. We found the redox potential of the H39M-b_5 protein to be −240 mV relative to the hydrogen electrode as compared to between +6 mV and −6 mV for wild-type cytochrome b_5, consistent with this interpretation.[10]

Another strong motivation for alteration of heme axial ligand environments is the possibility of generating a new protein with novel chemical properties. With an open heme cleft and possibility of ligand coordination, one might expect H39M-b_5 to have an increased peroxidase activity over the six-coordinate bis-imidazole-ligated wild-type protein. This was indeed found to be the case.[11] H39M-b_5 is also able to catalyze intermolecular oxidative chemistry as demonstrated by the hydrogen peroxide dependent oxidative demethylation of *N,N*-dimethylaniline.[12] Native cytochrome b_5 is completely inactive in this reaction, as expected for a six-coordinate electron transport protein.

In summary, we have described the first replacement of a heme protein ligand by site-directed mutagenesis. A b-type cytochrome with bis-imidazole axial ligands has been altered by replacement of one coordinating ligand to produce a hexacoordinate high-spin ferric protein. This new species has unique properties, drastically altered redox behavior, and is active in oxidative chemistry.

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Cooperative Binding by an Amphipathic Host

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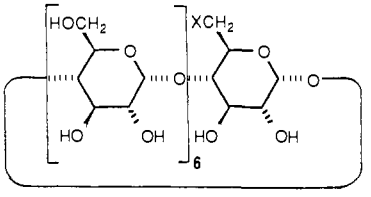
Allosteric regulation of binding and catalysis is a common feature of biochemical processes,[1] especially in the regulation of enzymes by molecular effectors. When affinity of enzyme for substrate increases with increasing effector concentration, the allostery is termed positive cooperativity, and the transition from the inactive to the active state of the protein is the allosteric transition. Allosteric transitions are typically transmitted by conformational changes,[2] though other mechanisms have also been advanced.[3] Positive cooperativity in the binding of inorganic guests to synthetic hosts has been observed in dicoronands linked by a biphenyl,[4] gable porphyrins,[5] porphyrin dimers,[6] and crystalline heme models.[7] Cooperativity has also been reported in micelle-catalyzed reactions.[8] We report here the synthesis of **4**, a new amphipathic host derived from β-cyclodextrin which exhibits positive cooperativity in the binding of simple organic guests in aqueous solution. The allosteric transition is apparently mediated by changes in aggregate morphology.



1 X = OH
2 X = OTs
3 X = $NH_3^+Cl^-$
4 X = $NH_2^+(CH_2)_{15}CH_3$ Cl^-

β-Cyclodextrin-6-monotosylate (**2**) was converted to **4** in 24% yield.[9] The association of 4-nitrophenol with **4** ([4-NP] = 0.36

(9) Harbury, H.; Cronin, J.; Fanger, M.; Hettinger, T.; Murphy, A.; Myer, Y.; Vinogradov, S. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *54*, 1658. Marchon, J.; Mashiku, T.; Reed, C. In *Electron Transport and Oxygen Utilization*; Ho, C., Ed.; Elsevier: North-Holland, New York, 1982; pp 67–73.

(10) The redox potential of H39M-b_5 was determined by the photoreduction technique with use of EDTA as electron donor, 9-aminoacridine as photosensitizer, and Safranine T (E_o' = −289 mV) as system potential indicator as described in the following: Sligar, S.; Gunsalus, I. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1079.

(11) Peroxidase activities were conducted with 1 nmol of cytochrome b_5 in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 220 nmol of hydrogen peroxide and 0.07 mg of *o*-dianisidine. An extinction coefficient of 1.13 × 10^4 cm^{-1} M^{-1} at 460 nm was used for product concentrations. Greater than 35 turnovers of H39M-b_5 were obtained with peroxidase activities for the various preparations of H39M-b_5, with the following activities, 4.61 U/μmol; wild-type b_5, 0.127 U/μmol protein; BSA–hemin (1:1), 0.379 U/μmol; hemin alone, 0.578 U/μmol; hemin–imidazole at ratios from 1:0.25 to 1:2, 0.729 U/μmol.

(12) Five nmol of H39M-b_5 in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 50–105 nmol of hydrogen peroxide and 500 nmoles of *N,N*-dimethylaniline as substrate, were allowed to react for 1 h at 25 °C. 1,2,4-trichlorobenzene was used as an internal standard. The reaction mixtures were extracted 3 times with chloroform and analyzed by gas chromatography on a Carbowax 20 M column with use of an HP5710A gas chromatograph and an HP3390A integrator. Reactions yielded 0.41 ± 0.03 nmol of *N*-methylaniline per nmol of hydrogen peroxide independent of the oxidant concentration corresponding to 4–8 turnovers of cytochrome.

(1) (a) Koshland, D. E. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1970; Vol. 1, p 342. (b) Fersht, A. In *Enzyme Structure and Mechanism*; W. H. Freeman: New York, 1985; pp 263–292.

(2) (a) Perutz, M. F. *Ann. Rev. Biochem.* **1979**, *48*, 327. (b) Monod, J.; Wyman, J.; Changeux, J.-P. *J. Mol. Biol.* **1965**, *12*, 88. (c) Koshland, D. E.; Nemēthy, G.; Filmer, D. *Biochemistry* **1966**, *5*, 365.

(3) (a) Cooper, A.; Dryden, D. T. F. *Eur. Biophys. J.* **1984**, *11*, 103. (b) Cornish-Bowden, A.; Cárdenas, M. L. *J. Theor. Biol.* **1987**, *124*, 1.

(4) (a) Rebek, J., Jr. *Acc. Chem. Res.* **1984**, *17*, 258. (b) Rebek, J., Jr.; Costello, T.; Marshall, L.; Wattley, R.; Gadwood, R. C.; Onan, K. *J. Am. Chem. Soc.* **1985**, *107*, 7481. (c) Rebek, J., Jr.; Wattley, R. V.; Costello, T.; Gadwood, R.; Marshall, L. *J. Am. Chem. Soc.* **1980**, *102*, 7400. (d) Rebek, J., Jr.; Trend, J. E.; Wattley, R. V.; Chakravorti, S. *J. Am. Chem. Soc.* **1979**, *101*, 4333. (e) Rebek, J., Jr.; Marshall, L. *J. Am. Chem. Soc.* **1983**, *105*, 6668.

(5) (a) Tabushi, I.; Kugimiya, S. *J. Am. Chem. Soc.* **1986**, *108*, 6926. (b) Tabushi, I.; Kugimiya, S.; Sasaki, T. *J. Am. Chem. Soc.* **1985**, *107*, 5159. (c) Tabushi, I.; Kugimiya, S.; Kinnaird, M. G.; Sasaki, T. *J. Am. Chem. Soc.* **1985**, *107*, 4192. (d) Tabushi, I.; Sasaki, T. *J. Am. Chem. Soc.* **1983**, *105*, 2901. (e) Tabushi, I.; Sasaki, T. *Tetrahedron Lett.* **1982**, *23*, 1913.

(6) (a) Traylor, T. G.; Tatsuno, Y.; Powell, D. W.; Cannon, J. B. *J. Chem. Soc., Chem. Commun.* **1977**, 732. (b) Traylor, T. G.; Mitchell, M. J.; Ciccone, J. P.; Nelson, S. *J. Am. Chem. Soc.* **1982**, *104*, 4986.

(7) Collman, J. P.; Brauman, J. I.; Rose, E.; Suslick, K. S. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 1052.

(8) (a) Piszkiewicz, D. *J. Am. Chem. Soc.* **1977**, *99*, 1550. (b) Piszkiewicz, D. *J. Am. Chem. Soc.* **1976**, *98*, 3053.

0002-7863/87/1509-7897$01.50/0