Communications to the Editor

Novel Axial Ligand Interchange in Cytochrome c: Incorporation of a Histidine at Position 82 Leads to Displacement of the Wild-Type Methionine-80 Ligand

Barton K. Hawkins,[†,‡] Sharon Hilgen-Willis,[§,‡,⊥] Gary J. Pielak,[*,∥] and John H. Dawson[*,†,∥]

Department of Chemistry & Biochemistry and School of Medicine, University of South Carolina Columbia, South Carolina 29208
Department of Chemistry and Program in Molecular Biology and Biotechnology University of North Carolina at Chapel Hill Chapel Hill, North Carolina 27599

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We herein demonstate, via near infrared magnetic circular dichroism (NIR-MCD) and electron paramagnetic resonance (EPR) spectroscopy, that replacement of Phe82 by His in an iso-1-cytochrome *c* mutant[1,2] from the yeast *Saccharomyces cervisiae* results in bis-His ligation of the heme iron. Because His82 is the only His on the distal side of the heme, it apparently coordinates the iron in place of the normal Met ligand even though the latter is still present in the mutant protein at position 80. This is an unprecedented example of a change in heme iron coordination structure achieved through site-directed mutagenesis in which *neither* of the original amino acid ligands has been replaced. Instead, mutagenesis of a residue two amino acids away from the wild-type distal ligand produces this interesting change in coordination structure. Exchanging one amino acid for another by site-directed mutagenesis has become a popular methodology in probing enzyme mechanisms. The change in coordination structure following mutagenesis presented herein, necessarily involving at least local peptide chain disordering, indicates that the presumption of identical active site topology or metal ligation following isometric amino acid replacement may not always be valid.

The NIR-MCD spectra (Figure 1) of ferric iso-1-cytochrome *c* with Cys102 replaced by Thr (C102T, employed herein as the wild-type[1]) and the double mutant also having Phe82 replaced by His (F82H) show only positive features characteristic of ferric low-spin heme proteins.[3] The NIR-MCD and corresponding EPR data are summarized in Table 1, along with those for horse cytochrome *c*, its imidazole complex, and additional bis-His and His/Lys ligated examples. The NIR-MCD spectra of ferric C102T and F82H,[4] as well as the UV–vis MCD in both ferric and ferrous states (data not shown),[5] compare well with those of native horse cytochrome *c* and its imidazole complex, respectively.[6] These observations are consistent with His/Met and bis-His ligation for the C102T and F82H variants, respectively.

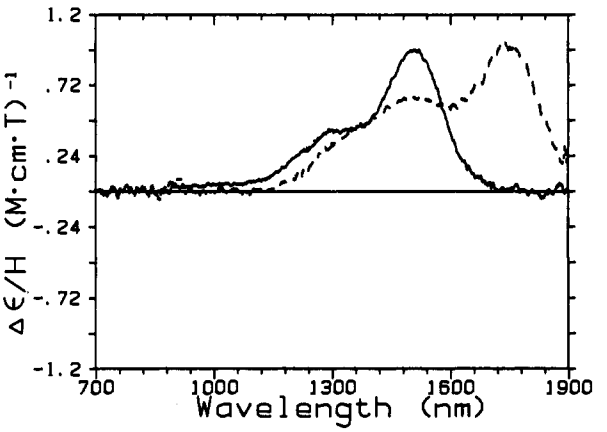

Figure 1. Near-IR magnetic circular dichroism spectra of 2.07 mM ferric F82H cytochrome *c* (—) and of 3.08 mM ferric C102T cytochrome *c* (– – –), obtained in 50 mM potassium phosphate buffer, pD 7.00 (corrected as observed pH + 0.44, ref 13), 277 K, 1.41 T, 0.1-cm path length. C102T is employed herein as the wild-type.

Replacement of the invariant surface residue, Phe, by Ser at position 82 results in formation of a solvent channel to the distal side of the heme, with Ser being oriented approximately in the same location relative to the heme as Phe82 is in the wild-type protein.[7] It is therefore possible that water or hydroxide is the heme iron distal ligand in F82H. However, all His/H_2O ligated heme iron proteins are at least partially high-spin at neutral pH; F82H is exclusively low-spin as indicated by the complete absence of a derivative-shaped MCD feature between 1000 and 1100 nm (9090–10 000 cm^{-1}) that is typical of high-spin ferric heme complexes.[8] Hydroxide (or other oxygen donor) ligated low-spin heme proteins exhibit E_{CT} between 9775 and 9300 cm^{-1},[4b] whereas

† Department of Chemistry & Biochemistry, University of South Carolina.
‡ Department of Chemistry and Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill.
∥ The School of Medicine, University of South Carolina.
‡ Submitted in partial fulfillment of the Ph.D. requirements at the University of South Carolina (B.K.H.) and the University of North Carolina at Chapel Hill (S.H.-W.).
⊥ Current address: Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104.

(1) (a) Our version of wild-type yeast iso-1-cytochrome *c*, the C102T variant,[1b] is structurally identical to the wild-type protein[1c] but is more amenable to biophysical studies.[1d] Variant proteins are described using the one-letter code[1e] with the original residue given first, followed by the residue number and the new residue. Throughout this paper, the numbering system for higher eukaryotic cytochromes *c* is used.[1f] (b) Cutler, R. L.; Pielak, G. J.; Mauk, A. G.; Smith, M. *Protein Eng.* **1987**, *1*, 95–99. (c) Gao, Y.; Boyd, J.; Williams, R. J. P.; Pielak, G. J. *Biochemistry* **1990**, *29*, 6994–7003. Gao, Y.; Boyd, J.; Pielak, G. J.; Williams, R. J. P. *Biochemistry* **1991**, *30*, 1928–1934. Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959–976. (d) Betz, S. F.; Pielak, G. J.; *Biochemistry*, **1992**, *31*, 12337–12344. (e) IUPAC-IUB. *J. Biol. Chem.* **1985**, *260*, 14–41. (f) Moore, G. R.; Pettigrew, G. W. *Cytochromes c: Evolutionary, Structural and Physiochemical Aspects*; Springer-Verlag: Berlin, 1990.

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(4) (a) Thomson and co-workers[4b–d] have shown that UV–vis MCD cannot be used to distinguish His/Met from bis-His ligated ferric porphyrins. However, the UV–vis MCDs of such adducts in the ferrous state differ significantly in the ratio of MCD signal intensities for the Soret region relative to the visible region. It therefore appears that UV–vis MCD can differentiate between these two ligand sets in the ferrous state. Further studies addressing this point are underway. (b) Gadsby, P. M. A.; Thomson, A. J. *J. Am. Chem. Soc.* **1990**, *112*, 5003–5011. (c) Thomson, A. J.; Gadsby, P. M. A. *J. Chem. Soc., Dalton Trans.* **1990**, 1921–1928. (d) Cheesman, M. R.; Greenwood, C.; Thomson, A. J. *Adv. Inorg. Chem.* **1990**, *36*, 201–255.

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Table 1. EPR Parameters and Energies of Near-IR Charge-Transfer Bands of Low-Spin Ferric Hemoproteins

protein	EPR g values[a]			peak wavelength of near-IR CT band[b] (nm)	E_{CT} (cm^{-1})[c]	ligation of heme
	g_z	g_y	g_x			
cytochrome c[d]	3.07	2.23	1.26	1725[e]	5797	His/Met
C102T[d]	3.10	2.25	1.35[f]	1725	5797	His/Met
F82H[d]	2.96	2.23	1.51[f]	1510	6623	His/His
cytochrome c-ImH[d]	2.96	2.26	1.51	1480[e]	6757	His/imidazole
cytochrome a[g]	3.03	2.21	1.45	1564	6394	His/His
cytochrome c_3[h]	3.02	2.25	1.50	1548	6460	His/His
cytochrome c_3[i]	2.96	2.29	1.59	1502	6658	His/His
cytochrome c-alk[j]	3.33	2.05	1.13[k]	1480	6757	His/Lys

[a] g values ordered as expected for typical heme iron complexes although no single-crystal experiments have been performed. [b] Peak wavelength of MCD at 277 K, 1.41 T for this work; MCD at 4.2 K, 5 T for work reported in ref 4b. [c] $E_{CT} = 10^7$(peak wavelength)$^{-1}$. [d] This work. Horse cytochrome c, cytochrome c-ImH; ImH, imidazole. C102T and F82H refer to the appropriate variant of yeast iso-1-cytochrome c, ref 1; C102T is employed herein as the wild-type. [e] These values are in excellent agreement with those reported in ref 4b. [f] Estimated from the relation $\sum g_i^2 = 16$, as the experimental location of the g value is uncertain owing to a low transition probability or the possibility of confusion with other signals. [g] Cytochrome a from cytochrome c oxidase complex, ref 4b. [h] Cytochrome c_3 Norway, pD 6.5, ref 4b. [i] *Desulphovibrio vulgaris* cytochrome c_3, ref 4b. [j] Alkaline horse cytochrome c (pD 11.0), ref 4b. [k] Experimental location of g value is uncertain because of low transition probability or to the possibility of confusion with other signals.

the value of E_{CT} observed for F82H is 6623 cm^{-1}. The NIR-MCD spectral properties of the F82H variant therefore rule out water, hydroxide, and other oxygen donors as the distal ligand to the heme iron.

Gadsby and Thomson have described a protocol for the identification of axial ligands in low-spin ferric protoporphyrin IX complexes.[4b-d] Comparison of NIR-MCD and EPR parameters for samples with unknown coordination to those of standards with known ligation leads, in general, to unambiguous axial ligand assignment. NIR-MCD *can* be used as the sole technique in distinguishing His/Met, bis-His, His/H_2O, His/OH, and His/Cys axial ligand sets.[9] However, NIR-MCD alone is not sufficient to differentiate bis-His from His/amine ligation.[4b-d] Alkaline horse cytochrome c (pD 11.0) is known to be His/Lys bound and exhibits a charge-transfer band (E_{CT}) at 6757 cm^{-1}, while the n-butylamine adduct of soybean leghemoglobin presents its transition at 6452 cm^{-1}.[4b] Thus the E_{CT} values for His/amine ligated examples overlap the range of 6135–6757 cm^{-1} (imidazole-bound leghemoglobin and horse cytochrome c–imidazole,[4b] respectively) observed for known *bis*-His adducts. The charge-transfer band for F82H (6623 cm^{-1}) lies within this range.

The NIR-MCD data (Table 1) provide strong exclusionary evidence concerning the distal ligand in the F82H variant; it is not Met, Cys, water, or hydroxide. This leaves Lys and His as candidates; His/Lys and bis-His complexes can readily be distinguished by EPR in conjunction with NIR-MCD using the Gadsby–Thomson protocol. F82H exhibits g values virtually identical to those of the imidazole complex of horse cytochrome c and in good agreement with those observed for several bis-His examples (Table 1). Furthermore, the EPR parameters observed for F82H are substantially different than those seen for horse cytochrome c (pD 11.0), known to have His/Lys heme ligands.[4b] Although some bis-His ligated systems have g_z as high as 3.55, these exhibit characteristic non-Gaussian features with a cutoff on the low-field edge leading to a ramp-shaped appearance for g_z,[4b] which is not seen for F82H. This evidence provides additional support for the conclusion that F82H is bis-His coordinated. The absence of Met ligation is also fully supported by ^1H NMR. No resonances indicative of Met ligation to the heme in either the ferrous or the ferric oxidation state were observed.[10]

In the absence of a three-dimensional structure for the F82H variant, it is not possible to rigorously define the geometry of the protein. However, there are no histidines on the distal side of the heme in the wild-type protein.[11] Residues –7 to 50[1a] are proximal to the heme; histidines 18 (proximal ligand), 26, 33, and 39 are thus all unavailable as distal ligands unless major conformational changes have occurred as a result of replacing Phe82 by His. Such changes, which would likely interdict formation of the necessary three-dimensional binding and electron-transfer domains, are unexpected.[12] Although mutagenesis of a residue two amino acids away from the wild-type ligand would not be expected to affect the heme iron coordination, the spectral evidence presented herein shows that the distal ligand of the F82H variant is His82 rather than Met80.

In summary, we have used the Gadsby–Thomson NIR-MCD/EPR protocol to determine that the heme iron of a novel cytochrome c, F82H, is ligated by His18 and His82. This novel ligation-state change must result from local disordering of the cytochrome c active site upon exchanging Phe82 for His. Thus, His82, a residue two amino acids away from the wild-type distal Met ligand, binds at the heme iron in F82H. As Phe and His are roughly the same size, this study provides an important caveat for site-directed mutagenesis studies, namely, local order may not be preserved even if the amino acids exchanged are of similar size. This point is particularly relevant for studies of metalloenzymes, in which, as demonstrated herein, the ligation state of the metal may be altered following site-directed mutagenesis.

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(9) A recent report[9b] questions the use of E_{CT}/EPR parameters to elucidate heme iron ligand sets as published by Thomson and co-workers.[4] A series of cytochromes c exhibits NMR features consistent with His/Met ligation, but EPR g values suggest bis-His ligation. However, the NMR experiments were conducted at 298 K and the EPR at 10 K. The apparent dichotomy raised by Teixeira *et al.* may be the result of changes in heme iron coordination upon freezing. Accordingly, NIR-MCD should be conducted at both temperatures to eliminate this possibility. (b) Teixeira, M.; Campos, A. P.; Aguiar, A. P.; Costa, H. S.; Santos, H.; Turner, D. L.; Xavier, A. *FEBS Lett.* **1993**, *317*, 233–236.

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(12) Further studies addressing the issue of heme ligation and activity *in vivo* are underway.

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