Studies of the Electronic and Dynamic Properties of High-Potential Iron Proteins by Substitution with Non-Natural Amino Acids. 3-Fluorotyrosine-Modified *Chromatium vinosum* High-Potential Iron Protein

Siu Man Lui and J. A. Cowan*,†

Evans Laboratory of Chemistry, The Ohio State University 120 West 18th Avenue, Columbus, Ohio 43210

Received January 18, 1994

Iron–sulfur clusters demonstrate a variety of redox, structural, and catalytic roles in proteins and enzymes,[1-6] and so they serve as useful paradigms for understanding the role of protein side chains in regulating the physicochemical properties of metalloredox prosthetic centers. Extensive studies of model cluster complexes, native ferredoxins, and high-potential iron proteins (HiPIPs) have greatly advanced the understanding of cluster chemistry in proteins.[7-13] To extend this work to systematic structure–function studies, we recently reported an efficient expression system for *Chromatium vinosum* HiPIP.[14] This affords us the opportunity to apply an array of molecular biology protocols (site-directed mutagenesis, isotope labeling, and incorporation of modified amino acids) both to explore the chemistry of the $[Fe_4S_4]$ prosthetic site and to examine the effect of systematic changes in structural and stereoelectronic factors that might regulate the physicochemical properties of protein-bound clusters. Here we describe the effect of replacing Tyr-19, a residue that lies in close proximity to the $[Fe_4S_4]$ core (Figure 1),[13a] with 3-fluorotyrosine (a structurally conservative replacement).[15] This strategy affords a convenient spectroscopic probe of the dynamics of ring motion, and electronic coupling between the $[Fe_4S_4]$ cluster and the aromatic ring.

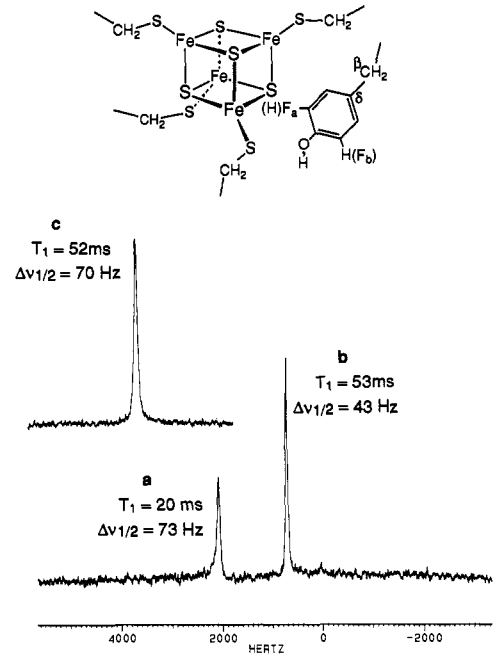

Figure 1. (Top) Schematic illustration of the putative π-complex formed between the reduced $[Fe_4S_4]$ cluster and Tyr-19. (Bottom) Representative ^{19}F NMR spectra obtained for reduced and oxidized HiPIP (5 mM in 200 mM NaCl, pH 6.5) after 400 and 750 scans, respectively, with a line broadening of 10 Hz. T_1 values are quoted for 25 °C. Resonance frequencies are relative to 3-fluorotyrosine obtained at pH 10 ($T_1 \sim 6$ s): a, 9.44 ppm; b, 3.05 ppm; c, 15.4 ppm.

The utility of ^{19}F NMR spectroscopy in studies of biological molecules has previously been demonstrated.[16,17] The ^{19}F NMR spectrum of reduced HiPIP (Figure 1) shows two peaks (a and b) in a 1:2 ratio (approximately), consistent with the two possible orientations of the ring face relative to the cluster (Figure 1). This result suggests restricted rotational motion of the ring around the C_β–C_γ bond in the reduced state.[18] Recent molecular dynamics and NOE studies have verified the proximity of the tyrosine and cluster, with only minor structural deviations from the crystallographic model.[9a] Ferricyanide oxidation produced a single downfield-shifted resonance (c), consistent with crystal-

† This work was supported by the NSF (CHE-8921468). J.A.C. is a Fellow of the Alfred P. Sloan Foundation and a National Science Foundation Young Investigator.

(1) Bartsch, R. G. *Methods Enzymol.* **1978**, *53*, 329–340.
(2) Emptage, M. H.; Kent, T. A.; Kennedy, M. C.; Beinert, H.; Munck, E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *80*, 4674.
(3) Telser, J.; Emptage, M. H.; Merlde, H.; Kennedy, M. C.; Beinert, H.; Hoffman, B. M. *J. Biol. Chem.* **1986**, *261*, 480.
(4) Higuchi, Y.; Yasuoka, N.; Kakudo, M.; Katsube, Y.; Yagi, T.; Inokuchi, H. *J. Biol. Chem.* **1987**, *262*, 2823–2826.
(5) Cunningham, R. P.; Asahara, H.; Bank, J. F.; Scholes, C. P.; Salerno, J. C.; Surerus, K.; Munk, E.; McCracken, J.; Peisach, J.; Emptage, M. H. *Biochemistry* **1989**, *10*, 4450.
(6) Langen, R.; Jensen, G. M.; Jacob, U.; Stephens, P. J.; Warshel, A. *J. Biol. Chem.* **1992**, *267*, 25625.
(7) (a) Holm, R. H. *Acc. Chem. Res.* **1977**, *10*, 427. (b) Ciurli, S.; Carrie, M.; Weigel, J. A.; Carney, M. J.; Stack, T. D. P.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 2654. (c) Carney, M. J.; Papaefthymiou, G. C.; Whitener, M. A.; Spartalian, K.; Frankel, R. B.; Holm, R. H. *Inorg. Chem.* **1988**, *27*, 346. (d) Laskowski, E. J.; Reynolds, J. G.; Frankel, R. B.; Foner, S.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1979**, *101*, 6562.
(8) (a) O'Sullivan, T.; Millar, M. M. *J. Am. Chem. Soc.* **1985**, *108*, 4096. (b) Christou, G.; Ridge, B.; Rydon, H. N. *J. Chem. Soc., Dalton Trans.* **1978**, 1423.
(9) (a) Banci, L.; Bertini, I.; Carloni, P.; Luchinat, C.; Orioli, P. L. *J. Am. Chem. Soc.* **1992**, *114*, 10683. (b) Bertini, I.; Capozzi, F.; Ciurli, S.; Luchinat, C.; Messori, L.; Piccioli, M. *Biochemistry* **1993**, *32*, 9387. (c) Bertini, I.; Ciurli, S.; Dikiy, A.; Luchinat, C. *J. Am. Chem. Soc.* **1993**, *115*, 12020.
(10) (a) Bertini, I.; Capozzi, F.; Ciurli, S.; Luchinat, C.; Messori, L.; Piccioli, M. *J. Am. Chem. Soc.* **1992**, *114*, 3332. (b) Bertini, I.; Banci, L.; Luchinat, C. *Struct. Bonding* **1990**, *72*, 113.
(11) (a) *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic Press; New York, 1973, Vols. 1 and 2; 1977, Vol. 3. (b) *Metal Ions in Biology*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1982; Vol. 4. (c) Sweeney, W. V.; Rabinowitz, J. C. *Annu. Rev. Biochem.* **1980**, *49*, 139. (d) Munk, E.; Papaefthymiou, V.; Sererus, K. K.; Girerd, J.-J. In *Metal Clusters in Proteins*; ACS Symposium Series; Que, L., Ed.; American Chemical Society: Washington, DC, 1988.
(12) (a) Nettesheim, D. G.; Harder, S. R.; Feinberg, B. A.; Otvos, J. D. *Biochemistry* **1992**, *31*, 1234. (b) Gaillard, J.; Albrand, J.-P.; Moulis, J.-M.; Wemmer, D. E. *Biochemistry* **1992**, *31*, 5632. (c) Krishnamoorthi, R.; Markley, J. R.; Cusanovich, M. A.; Prysiecki, C. T.; Meyer, T. E. *Biochemistry* **1986**, *25*, 60.
(13) (a) Carter, C. W., Jr. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic Press: New York, 1977; Vol. 3, pp 157–204. (b) Carter, C. W.; Kraut, J.; Freer, S. T.; Xuong, N. H.; Alden, R. A.; Bartsch, R. G. *J. Biol. Chem.* **1974**, *249*, 4212–4225. (c) Carter, C. W.; Kraut, J.; Freer, S. T.; Alden, R. A. *J. Biol. Chem.* **1974**, *249*, 6339–6346. (d) Palmer, G. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XII, Part B, pp 1–56.
(14) Agarwal, A.; Tan, J.; Eren, M.; Tevelev, A.; Lui, S. M.; Cowan, J. A. *Biochem. Biophys. Res. Commun.* **1994**, *197*, 1357–1362.

(15) 3-Fluorotyrosine modified HiPIP was obtained by growing an *Escherichia coli* cell line containing the HiPIP expression vector in a minimal medium supplemented with 100 mg/L of each amino acid (3-fluorotyrosine replacing Tyr).[14] Minor perturbations are noted in optical properties and high-field ^1H NMR. In the reduced state the paramagnetically-shifted resonances moved upfield (<0.5 ppm), but moved downfield (1–5 ppm) for oxidized protein.

(16) (a) Hull, W. E.; Sykes, B. D. *Biochemistry* **1974**, *13*, 3431. (b) Hull, W. E.; Sykes, B. D. *J. Mol. Biol.* **1975**, *98*, 121. (c) Hull, W. E.; Sykes, B. D. *J. Chem. Phys.* **1975**, *63*, 867. (d) Hull, W. E.; Sykes, B. D. *Biochemistry* **1976**, *15*, 1535.
(17) (a) Gerig, J. T.; McLeod, R. S. *J. Am. Chem. Soc.* **1973**, *95*, 5725. (b) Gerig, J. T.; Klinkenborg, J. C.; Nieman, R. A. *Biochemistry* **1983**, *22*, 2076. (c) Gamcsik, M. P.; Gerig, J. T.; Gregory, D. H. *Biochim. Biophys. Acta* **1987**, *912*, 303.

(18) The preference for one conformer may result from one of two factors. First, there may be a steric preference, since the C–F bond length is a little longer than that of C–H (1.26–1.41 Å relative to 1.06–1.10 Å).[19] Second, the polarity of the C–F bond, $C^{\delta+}$–$F^{\delta-}$, may promote a favorable interaction with the cluster.

(19) (a) Sharp, A. G. In *Carbon-Fluorine Compounds*; Elsevier: Amsterdam, 1972; p 33. (b) Gerig, J. T. In *Biological Magnetic Resonance*; Plenum: New York, 1978; Vol. 1, p 142.

0002-7863/94/1516-4483$04.50/0

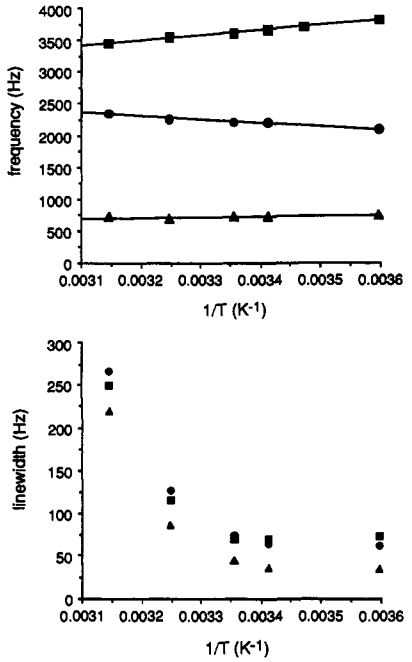

Figure 2. Temperature dependence of ^{19}F resonance frequencies (relative to 3-fluorotyrosine) and line widths: a (●), b (▲), c (■). The reference line width did not vary with temperature over the range employed.

lographic results that show movement of Tyr-19 away from the cluster in the oxidized state,[13] with increased rotational freedom. Figure 2 shows the temperature dependences of the resonance frequencies and line widths ($\Delta\nu_{1/2}$) for a, b, and c,[20] while relaxation times (T_1, 25 °C) are noted in Figure 1. For resonance c in the oxidized sample, the decrease in line width expected for faster rotation is likely to be offset at higher temperatures by a more substantial contribution from the paramagnetism of the cluster. At lower temperatures the line width of c does indeed decrease with rising temperature (up to 25 °C), but this is not observed for signals a and b in the reduced state.

The larger $\Delta\nu_{1/2}$ and shorter T_1 for resonance a, relative to b, are consistent with one of the H-3 ring protons of Tyr-19 lying closer to the cluster, as suggested by X-ray studies. However, these effects cannot be rationalized by a simple dipolar model since there is a scalar contribution resulting from electronic coupling of the cluster and tyrosine. For such protein samples, the relatively small H–F couplings of fluorotyrosine ($^3J_{HF} \sim 9$ Hz) do not contribute greatly to the observed line widths, while the influence of chemical shift anisotropy (CSA) in the presence of neighboring protons is also negligible.[17b,21] At lower temperatures, both the similarity in line widths ($\Delta\nu_{1/2}$) for ^{19}F resonances obtained in the oxidized and reduced forms, where the paramagnetism of the cluster is very different, and the absence of a correlation between the temperature profiles in Figure 2 suggest that $\Delta\nu_{1/2}$ is dominated by exchange broadening rather than the paramagnetism of the cluster. Using the resonance line width of free 3-fluorotyrosine as a reference, upper and lower limits for the rotational exchange rate (k_r) of the tyrosine ring in the reduced and oxidized proteins, respectively, were estimated from standard theory:[21,22] reduced, $k_r(25\ °C) < 50\ s^{-1}$; oxidized $k_r(25\ °C) > 7 \times 10^4\ s^{-1}$. These data are consistent with previous estimates of k_r for hindered rotation around the C_β–C_γ bond of a tyrosine side chain.[16b]

Electronic coupling of the cluster and tyrosine is also reflected by the positive change in cluster reduction potential ($\Delta\Delta E^{\circ\prime} \sim 30 \pm 10$ mV), which correlates with the shift for the $Y^{\bullet+}/Y$ couple (Y = Tyr or F-Tyr) ($\Delta\Delta E^{\circ\prime} \sim 37 \pm 10$ mV). The shift to a more positive potential is consistent with the stabilizing influence of the electron-withdrawing fluorine. While this result does not directly support a major role for tyrosine in defining the absolute potential of the cluster, it does indicate a substantial electronic coupling between these centers that may be of functional significance. Finally, these results confirm the use of fluorinated aromatic amino acids as useful probes of the electronic, structural, and dynamic properties of such residues in redox proteins.

(20) Since π–π interactions with the cluster are likely in the reduced form, the magnetic contribution could be dipolar or contact in origin. In the oxidized state the effect is likely to be dipolar.

(21) For low molecular weight proteins such as HiPIP ($M_r \sim 9600$), the contribution to line broadening from CSA is negligible (≤3 Hz)[16b] and has been omitted from the estimation of k_r noted below in footnote 22.

(22) The exchange rate (k_r) is defined by either $k_r \sim \pi(\Delta\nu_{1/2})$ or $k_r \sim \pi(\nu_x - \nu_y)^2/(2\Delta\nu_{1/2})$ in the slow and fast exchange limits, respectively; Harris, R. K. *Nuclear Magnetic Resonance Spectroscopy*; Longman: London, 1986; pp 119–131. For the latter, we take $\nu_x - \nu_y$, the difference frequency for the two environments in the slow exchange limit, to be equivalent to the value in the reduced state (~1500 Hz). This appears a reasonable estimation, given the minor influence of the paramagnetic cluster on fluorine chemical shift values.