Communications to the Editor

Direct Observation of Protein Vibrations by Selective Incorporation of Spectroscopically Observable Carbon−Deuterium Bonds in Cytochrome *c*

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The role of dynamics in the biological function of proteins and enzymes has remained a topic of intense debate for decades. A direct probe of specific protein vibrations would be enormously useful for understanding the dynamics associated with protein function, but such experimental methods have not been available.[1] The use of vibrational spectroscopy, which is the most direct method to study dynamics, has been limited by the congestion of the protein vibrational spectrum.[2] Previous studies have used isotopic labeling, in conjunction with difference Fourier transform infrared (FT IR) spectroscopy, to examine protein vibrations in the amide region of the IR spectrum.[3] These vibrations tend to be delocalized due to spectral overlap and large dipole−dipole coupling. Thus, such studies are most suitable for characterizing global properties of proteins, such as secondary structure, and less suitable for probing specific bond vibrations. Spectral congestion has also prohibited the selective excitation of unique protein vibrations, thereby limiting the use of conventional pump−probe experiments, which have been used to follow vibrational energy flow in smaller molecules.[4] Interestingly, protein vibrational spectra have a "transparent window" that is free of absorptions, between 1800 and 2600 cm^{-1}. Chromophores absorbing in this region are detectable even in the presence of high protein concentrations. Moreover, due to the absence of other protein vibrations in this energy range, absorptions in this transparent window are more "local mode" in character and correspond to specific bond vibrations.[5] Many studies have taken advantage of this window to examine the vibrations of small cofactor-bound ligand molecules, such as CO, NO, and N_3^-.[6] However, these studies do not directly observe the protein itself. Therefore, details of protein motion and dynamics have been probed only indirectly and have consequently remained obscure.

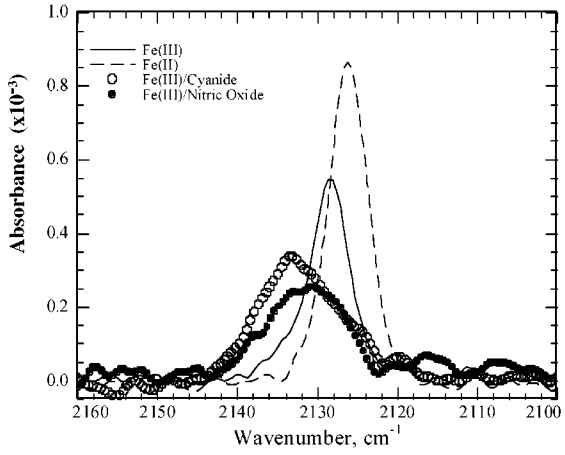

Figure 1. C−D absorption of (*methyl-d*$_3$)methionine labeled M65L cytochrome *c*: oxidized, pH 5 (solid line); reduced with ascorbic acid (dashed line); oxidized with Met80 displaced from heme Fe by the addition of $K^{13}C^{15}N$ (open circles); oxidized with Met80 displaced from heme Fe by the addition of nitric oxide (closed circles).

To observe specific protein local mode vibrations, we have introduced C−D bonds into horse heart cytochrome *c* (cyt *c*). The C−D stretching vibration, which absorbs at approximately 2100 cm^{-1}, has been used in small molecule studies as a spectroscopic probe of conformation and dynamics.[7] Herein, we describe our use of cyt *c* as a model system to demonstrate that individual protein vibrations may be directly observed by the incorporation of an isotopically labeled amino acid, and that these C−D vibrations are sensitive probes of the protein environment.

Cytochrome *c* functions as an electron carrier in the mitochondrial electron-transport chain using the Fe(II)-Fe(III) redox couple of a covalently attached heme prosthetic group. The protein has been well characterized by biophysical studies,[8] which have demonstrated that the protein environment plays an important role in controlling the redox state of the iron center. In particular, the Fe−S bond between the heme and the protein-based Met80 ligand is thought to play a critical role in the redox properties of the cofactor.[9] Met80 also plays an important role in folding, in which the final step requires this ligand to replace His33 at the iron center, a rearrangement that has been suggested to be strongly coupled to backbone folding.[10] IR experiments have been used to study hemoprotein ligands such as CO and NO, but experiments that observe protein based ligands such as Met80 in cyt *c* have not been possible, and the details of how this critical residue contributes to the biological functions or folding of this protein have remained obscure. We were therefore interested in the selective deuteration of this protein side-chain ligand to make its dynamics spectroscopically observable.

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(1) Fleming, G. R.; Hänggi, P. *Activated Barrier Crossing*; World Scientific: Singapore, 1993.

(2) (a) Baburina, I.; Moore, D. J.; Volkov, A.; Kahyaoglu, A.; Jordan, F.; Mendelsohn, R. *Biochemistry* **1996**, *35*, 10249−10255. (b) Bare, G. H.; Alben, J. O.; Bromberg, P. A. *Biochemistry* **1975**, *14*, 1578−1583.

(3) (a) Brauner, J. W.; Dugan, C.; Mendelsohn, R. *J. Am. Chem. Soc.* **2000**, *122*, 677−683. (b) Hutchison, R. S.; Betts, S. D.; Yocum, C. F.; Barry, B. A. *Biochemistry* **1998**, *37*, 5643−5653.

(4) (a) Kittrell, C. In *Molecular Dynamics and Spectroscopy by Stimulated Emission Pumping*; Dai, H.-L., Field, R. W., Eds.; World Scientific: Singapore, 1995. (b) Nesbitt, D. J. *J. Phys. Chem.* **1996**, *100*, 12735−12756. (c) Felker, P. M.; Zewail, A. H. In *Jet Spectroscopy and Molecular Dynamics*; Hollas, J. M., Phillips, D., Eds.; Blackie A&P: London, 1995. (d) McDonald, J. D. *Annu. Rev. Phys. Chem.* **1979**, *30*, 29.

(5) Wilson, E. B., Jr.; Decius, J. C.; Cross, P. C. *Molecular Vibrations*; Dover Publications: New York, 1955.

(6) See for example: (a) Anfinrud, P. A.; Han, D.; Hochstrasser, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8387−8391. (b) Laberge, M.; Vanderkooi, J. M.; Sharp, K. A. *J. Phys. Chem.* **1996**, *100*, 10793−10801. (c) Lian, T.; Locke, B.; Kholodenko, Y.; Hochstrasser, R. M. *J. Phys. Chem.* **1994**, *98*, 11648−11656. (d) Schelvis, J. P. M.; Kim, S.; Zhao, Y.; Marletta, M. A.; Babcock, G. T. *J. Am. Chem. Soc.* **1999**, *121*, 7797−7400. (e) Rector, K. D.; Rella, C. W.; Hill, J. R.; Kwok, A. S.; Sligar, S. G.; Chien, E. Y. T.; Dlott, D. D.; Fayer, M. D. *J. Phys. Chem. B* **1997**, *101*, 1468−1475.

(7) See for example: (a) Ohno, K.; Nomura, S.-I.; Yoshida, H.; Matsuura, H. *Spectrochim. Acta, Part A* **1999**, *55*, 2231−2246. (b) Grajar, L.; Baron, M. H.; Becourarn, S.; Czernecki, S.; Vallery, J. M.; Reiss, C. *Spectrochim. Acta* **1994**, *50A*, 1015−1022. (c) Dluhy, R. A.; Mendelsohn, R.; Casal, H. L.; Mantsch, H. H. *Biochemistry* **1983**, *22*, 1170−1177. (d) Pastrana, B.; Mautone, A. J.; Mendelsohn, R. *Biochemistry* **1991**, *30*, 10058−10064. (e) Echabe, I.; Requero, M. A.; Goñi, F. M.; Arrondo, J. L. R.; Alonso, A. *Eur. J. Biochem.* **1995**, *231, 199−203.* (f) Fraile, M. V.; Lopez-Rodriguez, G.; Gallego-Nicasio, J.; Carmona, P. *Biopolymers (Biospectroscopy)* **2000**, *57*, 11−18.

(8) Bertini, I.; Gray, H. B.; Lippard, S.; Valentine, J. *Bioinorganic Chemistry*; University Science Books: Sausalito, CA, 1994.

(9) (a) Raphael, A. L.; Gray, H. B. *J. Am. Chem. Soc.* **1991**, *113*, 1038. (b) Raphael, A. L.; Gray, H. B. *Proteins: Struct. Funct. Genet.* **1989**, *6*, 338.

(10) Colón, W.; Wakem, L. P.; Sherman, F.; Roder, H. *Biochemistry* **1997**, *36*, 12535−12541.

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Incorporation of (*methyl-d*$_3$)methionine (Cambridge Isotope Laboratories, Andover, MA) into cyt *c* was accomplished with minor modifications of literature protocols[11] and spectra were acquired as described in the Supporting Information. To evaluate the sensitivity of (*methyl-d*$_3$)Met80 to the heme environment, spectra of oxidized and reduced cyt *c* were examined (Figure 1). The C−D vibrations of Met80 proved to be sensitive to the oxidation state of the cofactor, shifting by 2.4 cm^{-1} to lower energy upon reduction (from 2128.6 to 2126.2 cm^{-1}), as shown in Figure 1. Structural studies of the homologous yeast iso-1-cyt *c* show that little structural reorganization takes place upon reduction; however, slight motion of protein residues and of an internally bound water molecule results in the formation of a hydrogen bond between the Tyr67 hydroxyl group and the Met80 S atom.[12] Brayer and co-workers have suggested that in the reduced state, the Tyr67-Met80 hydrogen bond increases the electron-withdrawing strength of the sulfur ligand, and that this effect is important for establishing the redox properties of the protein.[12] The observed shift in the C−D vibrations may also result from changes in the electron density centered at the Met80 sulfur atom due to hyperconjugation between C−D σ and sulfur d-orbitals. Alternatively, the reduced Fe(II)−S bond may contribute to the observed shift due to increased charge localization at sulfur[13a] and increased electron donation into C−D σ^* orbitals, similar to effects seen in small organic molecules.[14] The spectral shift might also result from a change in the polarity of the environment.[6b,15]

To further define the origin of this red-shift of the C−D bonds to lower energy upon reduction, we examined the IR spectrum of (*methyl-d*$_3$)Met80 Y67F cyt *c* (see Supporting Information). The oxidized wt and Y67F mutant proteins exhibited essentially identical spectra (the Y67F protein shows a 0.2 cm^{-1} blue-shift), implying that the C−D bonds of Met80 are not very sensitive to the loss of the tyrosine hydroxyl group in the oxidized protein. In the reduced protein, the mutation results in the replacement of the Met80-Tyr67 hydrogen bond with a Met80-water hydrogen bond,[16] and a 1.0 cm^{-1} red-shift is observed (from 2126.2 to 2125.2 cm^{-1}). If polarity changes were the dominant factor, both the oxidized and reduced forms of the mutant might be expected to show a similar shift in C−D frequency as only small changes in distances between these residues are observed in each form of the protein.[12,16] Therefore, it seems likely that the spectral red-shifts result from changes in Met80 through-bond interactions with the heme cofactor, other protein residues, or water molecules.

To further evaluate the sensitivity of (*methyl-d*$_3$)Met80, we displaced it from the Fe(III) center with the stronger ligands cyanide and nitric oxide (Figure 1). Addition of the negatively charged cyanide ligand to the oxidized protein caused ν_{CD} to blue-shift to higher energy by 3.5 cm^{-1} (from 2128.6 to 2132.1 cm^{-1}). Addition of the neutral nitric oxide ligand to the same protein also caused ν_{CD} to shift to higher energy by 2.7 cm^{-1} (from 2128.6 to 2131.3 cm^{-1}). The blue-shift of the peak upon Fe−S cleavage with either a negative or a neutral ligand is particularly noteworthy.

The above data likely reflect specific changes in the Met80 environment, including hydrogen bonding and electrostatics. However, it is possible to draw some general conclusions. Either reduction of the heme group or addition of cyanide to the oxidized protein introduces a formal negative charge, while addition of neutral nitric oxide preserves the formal charge. Reduction results in an increase in the strength of the Met80-Fe bond[13] and introduces a hydrogen bond with Tyr67, while addition of either ligand cleaves the Fe−S bond. The blue-shift observed upon cleaving the Fe−S bond with isostructural, but differently charged ligands, and the opposite red-shift observed upon strengthening the same bond by reduction, imply that short-range interactions between sulfur and the iron center, or other protein residues, are more important than the overall electrostatic field at Met80. The Y67F data are consistent with this conclusion. The removal of the Tyr67 hydroxyl group appears to affect the Met80 environment only when the residues are in direct contact. These data indicate that the Met80 CD_3 vibrations may be primarily sensitive to hyperconjugative interactions with sulfur-based orbitals. These interactions depend on electronic properties of the sulfur atom and are fixed by "through-bond" interactions, such as the strength of the Fe−S bond or the strength and number of hydrogen bonds to other protein residues, rather than by "through-space" interactions.

Many experiments have taken advantage of CO, NO, or N_3^- absorption in the transparent region of the protein IR spectrum and much has been learned regarding ligand geometry and dynamics. However, these experiments yield only indirect and incomplete information about the protein. In contrast, C−D labeled amino acids report directly on the protein. Due to the absence of other protein vibrations of similar energy, C−D vibrations are more local in character and correspond to specific protein vibrations. The C−D IR vibrations of (*methyl-d*$_3$)Met80 labeled cyt *c* are easily observed and sensitive to the protein environment. Future studies will focus on using C−D labels to further understand the structural and dynamic details of how cyt *c* uses Met80 to control redox activity and protein folding. Finally, the availability of C−D absorptions associated with known protein motions should allow for the excitation of given protein vibrations and may facilitate protein pump−probe experiments in these biological systems. The method should prove to be quite general, and further studies, including deuteration of other residues in cyt *c*, or other proteins of interest, should complement the large body of literature focusing on ligand geometry and dynamics, by directly probing protein vibrations.

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Supporting Information Available: (*methyl-d*$_3$)Methionine FT IR spectra, oxidized and reduced Y67F cyt *c* FT IR spectra, tabulated peak data, and ES MS protein characterizations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Morar, A. S.; Kakouras, D.; Young, G. B.; Boyd, J.; Pielak, G. J. *J. Biol. Inorg. Chem.* **1965**, *4*, 220−222. See also Supporting Information.
(12) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959−976.
(13) (a) Rovira, C.; Carloni, P.; Parrinello, M. *J. Phys. Chem. B* **1999**, *103*, 7031−7035. (b) Tezcan, F. A.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1998**, *120*, 13383−13388.
(14) Nakai, H.; Kawai, M. *J. Chem. Phys.* **2000**, *113*, 2168−2174.
(15) (a) Phillips, G. N., Jr.; Teodoro, M. L.; Li, T.; Smith, B.; Olsen, J. S. *J. Phys. Chem. B* **1999**, *103*, 8817−8829. (b) Park, E. S.; Andrews, S. S.; Hu, R. B.; Boxer, S. G. *J. Phys. Chem. B.* **1999**, *103*, 9813−9817.
(16) Berghuis, A. M.; Guillemette, J. G.; Smith, M.; Brayer, G. D. *J. Mol. Biol.* **1994**, *235*, 1326−1341.