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Efforts toward Developing Direct Probes of Protein Dynamics

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A direct probe of specific protein vibrations would be of great value for the study of protein dynamics, but such probes have not been available.[1] The use of conventional vibrational spectroscopy has been limited by the congestion of the protein vibrational spectrum. Previous studies have used isotopic labeling and difference Fourier transform infrared (FTIR) spectroscopy to examine amide vibrations.[2] Other studies have taken advantage of protein-bound ligands or nitrile-derivatized amino acids, which absorb in a region of the IR spectrum unobscured by other protein vibrations (1900−2300 cm^{-1}).[3] However, these studies do not directly probe protein vibrations.

We have been developing a technique to directly study specific protein vibrations by synthesizing proteins that are site-specifically labeled with carbon−deuterium (C−D) bonds. Like amide bonds, C−D bonds report directly on protein dynamics, but they absorb in a region of the IR spectrum free of other protein absorptions (∼2100 cm^{-1}). While C−D bonds may be adiabatically decoupled from the majority of other protein vibrations, they are likely to possess specific low order resonances that facilitate IVR, as has been observed for C−H bonds.[4] Thus, it is unclear whether C−D line widths will be more sensitive to homogeneous (e.g., intramolecular vibrational energy redistribution (IVR) and/or pure dephasing) or inhomogeneous (e.g., conformational heterogeneity) effects (see Supporting Information). This is especially interesting considering the potential contribution of directed IVR and the possibility that proteins may have evolved to possess dynamics that facilitate their function.

We[5] and others[6] have used horse heart cytochrome *c* (cyt *c*) as a model system for studying protein dynamics. The Fe−S bond between the heme and the protein-based Met80 ligand is thought to play a critical role in both the folding and redox properties of the protein (Figure 1). We have site-specifically synthesized cyt *c* with Met80 methyl-d_3 (Met80-d_3) and shown that the CD_3 vibrations are sensitive to both the oxidation and folded state of the protein.[5] Here, we synthesized site-specifically labeled Met80-d_1 cyt *c* and characterized its C−D stretching absorption. This is the first characterization of a specific C−H/D bond within a protein, and its comparison with methionine methyl-d_1/d_3 and cyt *c* Met80-d_3 suggests that the probes should be useful for measuring both IVR and conformational heterogeneity within a protein.

Boc-methionine methyl-d_1 (Met-d_1) was prepared from the methyl ester of N^{α}-Boc-homocysteine and ICH_2D, as described in the Supporting Information, and the protected amino acid was incorporated into cyt *c* at position 80, as described previously.[5c,7] Spectra were recorded with 10 mM cyt *c* or 100 mM methionine in 100 mM NaOAc, pH 5, at room temperature on a Bruker Equinox 55 FT-IR with a 50 μm path length. In principle, two asymmetric vibrations and one symmetric vibration are predicted for a CD_3 group of C_s or C_1 symmetry. Correspondingly, in methionine-d_3, two overlapping absorptions and one, stronger, lower frequency absorption are apparent (Table 1). On the basis of ab initio calculations (Supporting Information), we assign the low frequency absorption to the symmetric stretch and the high frequency absorptions to overlapping asymmetric vibrations. In Met80-d_3, the symmetric stretch was again apparent, but the asymmetric stretches could not be resolved (Table 1). For the d_1-labeled species, three methyl group rotamers are expected to give rise to three absorptions in the chiral protein environment: two diastereotopic gauche (relative to the S−C_γ bond) and one anti absorption. Correspondingly, we observed three overlapping absorptions in methionine-d_1 and Met80-d_1 (Table 1 and Figure 1). On the basis of ab initio calculations, we assign the two lower frequency absorptions to gauche rotamers and the higher frequency absorption to the anti rotamer.

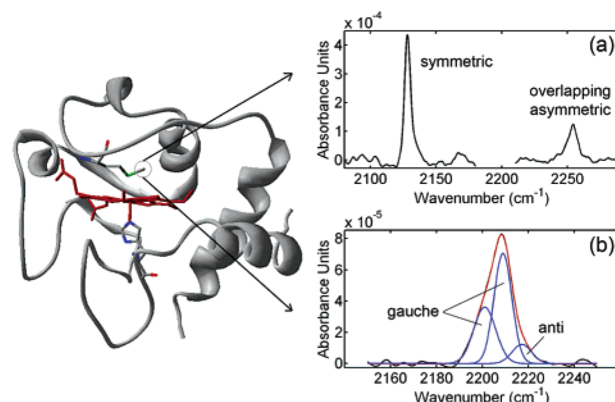

Figure 1. Labeled Met80 in cyt *c* (PDB: 1CRC). (a) IR spectrum of Met80-d_3. (b) IR spectrum of Met80-d_1.

When comparing the spectra, three conclusions are evident. First, all of the C−D absorptions are significantly more narrow in the protein than in the free amino acid. Second, in both the free amino acid and the protein, the CD_1 absorptions are not more narrow than the CD_3 absorptions. Third, the symmetric CD_3 stretch is significantly narrower than either of the (overlapping) asymmetric CD_3 or CD_1 stretches. To better understand these observations, we used Voigt functions to deconvolute the observed line widths (Γ_{obs}) into Lorentzian (homogeneous, Γ_{hom}) and Gaussian (inhomogeneous, Γ_{inh}) contributions (Table 1).[8] The dominance of Γ_{inh} over Γ_{hom}, along with the narrowing associated with transfer to the protein environment, suggests that the CD_1 and asymmetric CD_3 absorptions are primarily inhomogeneously broadened. Thus, we conclude that cyt *c* provides a more restricted environment with less conformational heterogeneity than is present with the free amino acid in solution. The analysis also suggests that the symmetric CD_3 line

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Table 1. Experimental Data. Standard Error Means are at the 95% Confidence Level

		ν_{obs} (cm^{-1})	Γ_{obs} (cm^{-1})	max Γ_{inh} (cm^{-1})	min Γ_{hom} (cm^{-1})
Met-d_1	anti	2211.4 ± 0.5	16.5 ± 1.3	14	4.2
	gauche	2200.4 ± 2.0	29.6 ± 7.0	25	1.3
	gauche	2188.1 ± 3.4	27.7 ± 2.4	25	4.5
Met80-d_1	anti	2217.2 ± 4.3	9.8 ± 0.7	12	0.7
	gauche	2208.9 ± 0.5	9.1 ± 1.8	8.3	0.2
	gauche	2201.1 ± 1.8	11.1 ± 3.5	12	0.3
Met-d_3	asym′	2245.1 ± 0.1	13.3 ± 0.1	13	1.0
	asym″	2244.5 ± 0.3	21.6 ± 0.2	20	1.5
	sym	2135.2 ± 0.1	9.6 ± 0.5	4.9	5.7
Met80-d_3	asym′, asym″	2254.5 ± 0.4	10.1 ± 1.5		
	sym	2128.0 ± 0.2	5.5 ± 0.2	5.4	0.3

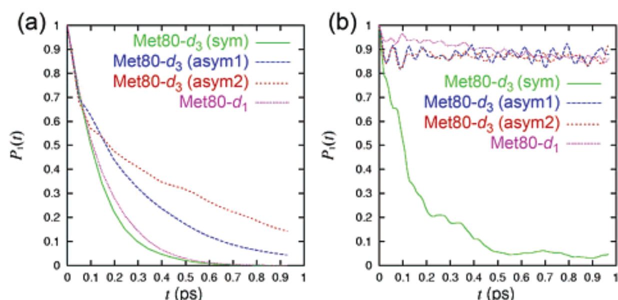

Figure 2. Population time evolution of (a) Met80 with a CHARMM potential at 300 K, and (b) methionine with an ab initio potential at 0 K.

width contains a more significant contribution from homogeneous broadening, $\Gamma_{inh} \sim \Gamma_{hom}$, than the other C−D absorptions (although Γ_{obs} is more narrow due to a decrease in Γ_{inh}). In this case, Γ_{hom} can be used to approximate the vibrational relaxation lifetime of the symmetric stretch (Supporting Information), which is found to be 0.6−0.9 ps in the free amino acid and between 1.8 and 18 ps in the protein.

To support these conclusions, we employed a theoretical method based on time-dependent perturbation theory.[9] The total Hamiltonian is divided into a system mode and the remaining bath modes and relaxation between the system and bath are mediated by 3rd and higher order couplings in the potential energy. The relaxation of excited state population was directly modeled, and T_1 was extracted from the behavior of the density matrix. By examining the contribution of each combination of bath modes, IVR mechanisms that mediate protein dynamics are clarified.[9,10]

When this method was applied to Met80-d_3 in cyt *c* using an empirical CHARMM potential,[10] we observed biphasic behavior for both asymmetric modes, with time constants of 0.15 and 0.5 ps (Figure 2a).[11] However, we observed single exponential kinetics with the symmetric stretch, with the entire amplitude decaying on the faster 0.15 ps time scale. To further examine the specific mode couplings that might mediate IVR, we used an ab initio surface (B3LYP/6-31+G(d,p)) to model vibrational relaxation in the free amino acid (Figure 2b). The differences in mode relaxation were even more pronounced, with the symmetric mode showing a fast single exponential decay with a time constant of 0.1 ps, and the asymmetric modes showing essentially no decay on the 1 ps time scale.

We observed Fermi resonances for all the C−D stretches, but their contributions to relaxation were small, except for the symmetric stretch of Met-d_3 (Supporting Information), where we observed a stronger 2:1 Fermi resonance with a C−D bending overtone. This Fermi resonance creates an IVR pathway that facilitates the fast relaxation of the excited symmetric stretch. As the 3rd order coupling elements are all similar, the strong Fermi resonance between the symmetric stretching and bending mode is primarily a frequency matching (resonance) effect. This specific low order resonance may explain the line width and line shape observed for this vibration.

C−D bonds incorporated throughout a protein provide an IR probe to directly characterize protein dynamics with high structural and temporal resolution. This study suggests that the stretching absorptions of C−D bonds incorporated at the methyl group of cyt *c* Met80 are predominantly inhomogeneously broadened. This was also found previously for CO bound to myoglobin,[3] which suggests that both probes report on the conformational heterogeneity of the protein. However, in the case of the symmetric stretch of the CD_3 group, there appears to be a contribution from homogeneous broadening. This vibration was also predicted to have the shortest lifetime due to a strong Fermi resonance. This suggests that this vibration might be useful for the study of IVR within the protein. Further studies, including theory and time-resolved spectroscopy, should further clarify these issues and begin to elucidate the mechanism of IVR within a protein, as well as the potential existence of IVR pathways and their contribution to protein dynamics and function.

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Supporting Information Available: Experimental and computational procedures and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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