Enzyme Catalysis

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Efforts Toward the Direct Experimental Characterization of Enzyme Microenvironments: Tyrosine100 in Dihydrofolate Reductase**

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The enzyme dihydrofolate reductase (DHFR), which catalyzes hydride transfer from the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to 7,8-dihydrofolate to produce tetrahydrofolate, has emerged as a paradigm for the study of enzyme catalysis.[1–3] It has been suggested that electrostatic complementarity between the enzyme and the transition state for hydride transfer contributes significantly to catalysis,[4–7] and computational studies have identified a number of residues that may mediate these interactions.[5,7] One of the most important is Tyr100, which directly contacts the nicotinamide hydride donor (Figure 1) and is thought to stabilize the developing positive charge on the cofactor in the hydride-transfer transition state. However, protein dynamics have also been suggested to contribute to DHFR catalysis through the population of rare but reactive substrate conformations.[8–12]

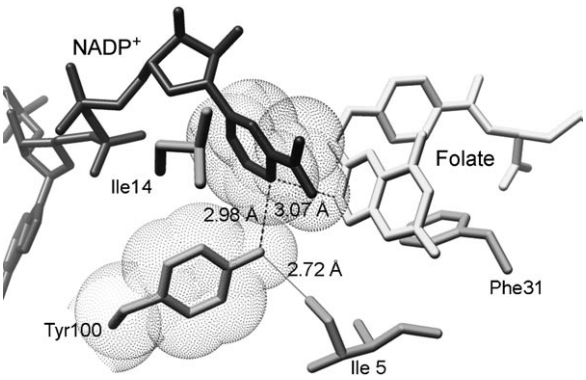

Figure 1. Structure of folate and $NADP^+$ bound to DHFR (PDB ID 1rx2) with side chains of Tyr100, Ile14, and Phe31, as well as Ile5 shown. Molecular graphics images were produced using the UCSF Chimera package.[28]

Vibrational spectroscopy provides a direct and bond-specific approach to the characterization of the microenvironments and motions of molecules, but with proteins its application is limited by congestion in the spectra. Previous approaches to observe individual vibrations, such as those associated with the amide backbone, sulfhydryl or carboxyl side chains, or bound water molecules, have used heavy atom isotope labeling and difference Fourier transform infrared (FTIR) spectroscopy.[13,14] In some cases, changes in the difference spectra have even been time-resolved.[14,15] However, the linewidths and frequencies of the absorptions are often difficult to deconvolute, as they remain in a congested region of the spectrum, and they are even more difficult to interpret in terms of specific protein motions, because of coupling with other vibrations. As part of a program to develop general probes of protein microenvironments and dynamics we have developed the use of carbon–deuterium (C–D) bonds as FTIR probes.[16–24] C–D bonds are sensitive to their environment and may be incorporated anywhere throughout a protein. While they are weaker than the other endogenous chromophores, their detection and analysis are facilitated by their unique absorption in an otherwise transparent region (ca. 2100 cm^{-1}) of the protein IR spectrum.

In principle, the C–D-based FTIR technique may be applied to a protein of any size. However, the available methods to site-selectively deuterate a protein are limited to synthesis or semisynthesis unless the amino acid of interest is present at only a single position. These limitations preclude the general application of the technique to many proteins, including DHFR, unless specific residues are made unique by site-directed mutagenesis. This latter approach has been applied to DHFR in a previous study, wherein all but one methionine residue was mutagenized to leucine to allow for site-specific labeling.[23] To examine a residue such as Tyr100 in DHFR without the introduction of potentially perturbative mutations we have used a biosynthetic method to site-selectively incorporate a photocaged, deuterated amino acid, which after photolysis yields the site-selectively deuterated, but otherwise natural, protein.

In previous studies, *o*-nitrobenzyl-*O*-tyrosine (ONBY), a tyrosine derivative protected with a photolabile *o*-nitrobenzyl group, was genetically encoded in *E. coli* by using an orthogonal $tRNA^{Tyr}_{CUA}$/aminoacyl-tRNA synthetase pair.[25,26] This unnatural amino acid was efficiently and site-specifically incorporated into proteins in response to an amber stop codon (TAG), which may be introduced into any gene of interest at any desired position by site-directed mutagenesis. For an initial characterization of the microenvironments and

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dynamics of DHFR, we used this approach to incorporate [2,3,4,5-D_4]Tyr into DHFR at Tyr100 and Tyr111. In contrast to Tyr100, Tyr111 is distal to the binding pocket and solvent exposed, and was thus chosen to serve as a control. OBNY-protected [D_4]Tyr100 and [D_4]Tyr111 DHFR were expressed in *E. coli* and purified as described in the Supporting Information. After purification, deprotection proceeded quantitatively in 40 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, upon exposure for 10 minutes to light with a wavelength of 360 nm to afford 20 and 26 mg L^{-1}, respectively, of [D_4]Tyr100 and [D_4]Tyr111 DHFR, as confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ESI mass spectrometry (see the Supporting Information). Protected and deprotected [D_4]Tyr111 DHFR showed wild-type activity, while [D_4]Tyr100 DHFR showed wild-type activity only after deprotection (see the Supporting Information).

We first characterized the C–D absorptions of protonated and deprotonated [2,3,4,5-D_4]tyrosine (Cambridge Isotopes) in 1M HCL or 1M NaOH, respectively. Both spectra show overlapping absorptions around 2200–2300 cm^{-1} that were fit to two Gaussian functions and assigned to C–D stretching modes (see the Supporting Information). As four C–D stretching modes are expected, we conclude that either two pairs of absorption bands are too overlapped to be resolved or two bands are too low in intensity to be observed. At both low and high pH values, the two absorptions have similar linewidths of about 20 cm^{-1}; but in alkaline solution the absorptions are blue-shifted by 15 to 17 cm^{-1} and the relative amplitudes are shifted to favor the high-frequency component.

To characterize the specific microenvironments and dynamics of DHFR, and how they might change during catalysis, we characterized the apoenzyme and the holoenzyme (bound NADPH) as well as complexes with folate and $NADP^+$, MTX and NADPH, or with folate alone. These complexes are thought to mimic the Michaelis complex, the transition state, and the product complex, respectively.[2] Similar to the free amino acid, in each case the IR spectrum of [D_4]Tyr111 DHFR showed overlapping absorptions around 2200–2300 cm^{-1}, which again are assigned as C–D stretching modes (Table 1 and Figure 2). The spectra are comprised of two dominant absorptions with relative frequencies and amplitudes similar to the deprotonated amino acid. However, while fitting the spectra required three Gaussian functions (see the Supporting Information), the frequencies and linewidths of the dominant absorptions did not change upon the addition of any of the ligands.

The spectra of apo as well as NADPH- and MTX/NADPH-bound [D_4]Tyr100 DHFR were also similar to those observed with the free amino acid, and well fit by two Gaussian functions (Table 1, Figure 2, and see the Supporting Information). Only small differences were observed in the three [D_4]Tyr100 spectra, with each showing absorption bands around 2247 and 2267 cm^{-1} with linewidths of about 15 cm^{-1}. In contrast, the [D_4]Tyr100 spectra of the folate and folate/$NADP^+$ complexes were dramatically different from the spectrum of the apo enzyme, as well as the other complexes. While the spectrum of the folate complex was well fit by two Gaussian functions at approximately 2247 cm^{-1} and 2266 cm^{-1} (see the Supporting Information), in contrast to the other complexes, the relative amplitudes shift significantly to favor the high-frequency absorption, which is also significantly broadened. The changes in amplitude resemble those induced by deprotonation of the free amino acid (see above), thus suggesting that Tyr100 is more strongly hydrogen-bonded in the folate complex. This hypothesis is consistent with crystallographic studies which reveal a hydrogen bond between the Tyr OH group and the carbonyl backbone of Ile5 that is uniquely short in the folate complex.[2] Furthermore, we observed a correlation between the length of this hydrogen bond in the different structures[2] and the relative intensities of the low- and high-frequency absorptions, further supporting this interpretation.

Interestingly, three absorptions are clearly apparent in the [D_4]Tyr100 spectra of the folate/$NADP^+$ complex (Table 1, Figure 2, and see the Supporting Information). Two of the absorptions, with frequencies at 2246 and 2263 cm^{-1}, are

Table 1: Spectroscopic data.

	Apo	NADPH	Folate/$NADP^+$	MTX/NADPH	Folate
[D_4]Tyr100[a]					
ν_A [cm^{-1}]	2247.0 ± 1.1	2247.4 ± 0.8	2246.0 ± 0.3	2247.0 ± 0.3	2246.7 ± 0.4
$FWHM_A$ [cm^{-1}]	16.8 ± 2.4	14.5 ± 0.8	13.4 ± 0.9	16.0 ± 0.5	13.6 ± 0.6
ν_B [cm^{-1}]	2266.6 ± 0.6	2267.6 ± 0.7	2262.6 ± 0.7	2269.0 ± 0.3	2265.7 ± 0.9
$FWHM_B$ [cm^{-1}]	18.8 ± 0.7	14.5 ± 1.9	19.4 ± 2.2	14.1 ± 1.8	26.6 ± 0.5
ν_C [cm^{-1}]			2278.9 ± 1.0		
$FWHM_C$ [cm^{-1}]			14.9 ± 0.5		
[D_4]Tyr111[a,b]					
ν_A [cm^{-1}]	2253.4 ± 0.3	2253.5 ± 0.2	2253.3 ± 0.7	2252.5 ± 0.3	2253.7 ± 0.2
$FWHM_A$ [cm^{-1}]	19.1 ± 1.8	18.8 ± 0.6	21.6 ± 1.1	18.3 ± 0.7	17.6 ± 0.4
ν_B [cm^{-1}]	2276.0 ± 0.8	2278.0 ± 0.8	2275.9 ± 1.9	2269.0 ± 1.9	2276.1 ± 1.9
$FWHM_B$ [cm^{-1}]	20.6 ± 0.8	19.3 ± 1.4	21.2 ± 3.2	28.6 ± 0.4	20.7 ± 2.0

[a] The two absorptions observed in the apo enzyme and in each complex are labeled A and B. The third absorption observed only in the folate/$NADP^+$ complex is labeled C. ν and FHHM correspond to the center frequency and full-width at half maximum linewidth, respectively. See text for details. [b] The frequencies and linewidths result from fits of the two dominant absorptions.

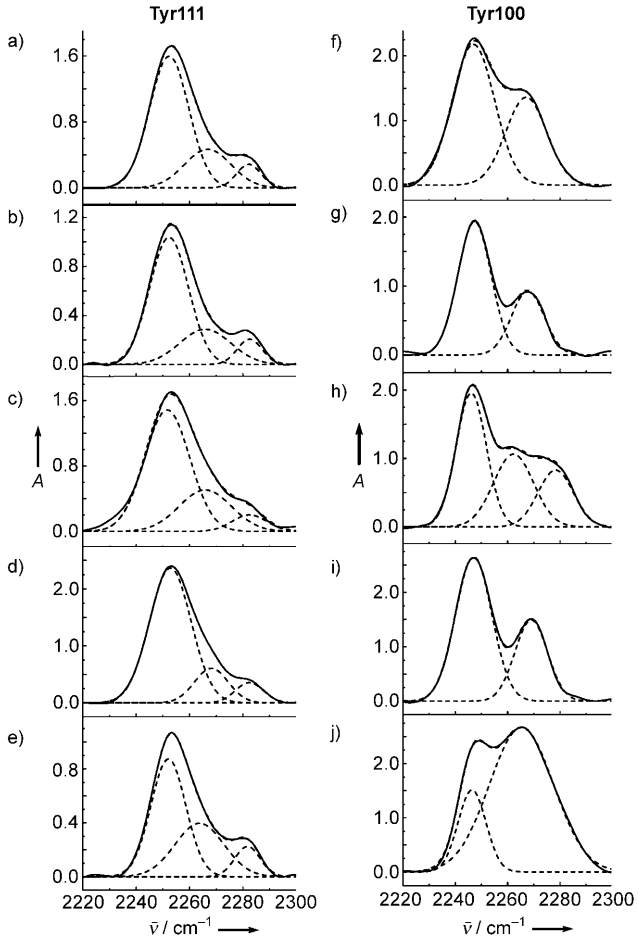

Figure 2. Spectra and fits of [D_4]Tyr111 (left) and [D_4]Tyr100 (right). a,f) Apo DHFR, b,g) NAFPH complex, c,h) folate/$NADP^+$ complex, d,i) MTX/NADPH complex, e,j) folate complex.

similar in relative amplitude, frequency, and linewidth to those observed in the spectrum of the apo as well as the NADPH- and MTX/NADPH-bound enzymes. This finding suggests that in the folate/$NADP^+$ complex, Tyr100 experiences an environment that is similar to that experienced in the apo enzyme and the other complexes. However, the additional high-frequency absorption at 2279 cm^{-1} is unique and must reflect the population of a unique microenvironment at Tyr100. Since the unique environment is not observed in the MTX/NADPH complex (where analogous ligands are bound and the protein assumes the same conformation[2]), it likely results from the charge on the cofactor. While this clearly indicates a strong electrostatic coupling between $NADP^+$ and Tyr100, a contribution of dynamics to the population of the unique environment cannot be excluded. In fact, NMR experiments detect a significant exchange term for Tyr100 that is unique to the $NADP^+$/folate complex,[27] thereby supporting the idea that the unique spectrum of [D_4]Tyr100 in this complex results, at least in part, from unique motions.

Structural and computational data have suggested that the hydroxy group of Tyr100 electrostatically stabilizes the developing positive charge at C4 of the nicotinamide moiety in the transition state. Our data clearly provide strong experimental support for this mechanism of catalysis. While the data also suggest that the dynamics of Tyr100 may change in the Michaelis complex, thus providing a mechanism for relaying more distal correlated motions to the reaction coordinate—which is thought to facilitate the population of reactive conformations[8–11]—additional studies are required to test this idea more directly. Nonetheless, given the tight packing between the Tyr OH group and the hydride donor (the heavy atoms are separated by a distance of only 3.0 Å, Figure 1), it seems likely that the motions of Tyr100 affect not only the stability of the developing charge, but also the geometry of the reaction coordinate, and that both effects might contribute to catalysis. The C–D-based technique is well suited to characterize enzyme microenvironments, including electrostatics and hydrogen bonding, as well as dynamics, and how each may contribute to function. Finally, additional advances in the biosynthetic methodology employed here should allow for the extension of the technique to the characterization of other important residues in DHFR, and other proteins as well.

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