Isotopic Labeling of Formate Dehydrogenase Perturbs the Protein Dynamics

Chethya Ranasinghe,[†,1] Philip Pagano,[†,1] Paul J. Sapienza,[‡] Andrew L. Lee,[‡] Amnon Kohen[†] and Christopher M. Cheatum,[†*]

[†]Department of Chemistry, University of Iowa, Iowa City, IA 52242-1727, USA

[‡]Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA

[*]To whom correspondence should be addressed; email: christopher-cheatum@uiowa.edu
[1]These authors contributed equally to this work.

Abstract

Isotope substitution of enzymes has become a means of addressing the participation of protein motions in enzyme-catalyzed reactions. The idea is that only the enzyme mass will be altered and not the electrostatics, so that the protein dynamics are essentially the same but at lower frequencies due to the mass change. In this study, we variably label all carbon atoms in formate dehydrogenase with ^{13}C, all nitrogen atoms with ^{15}N, and all non-exchangeable hydrogen atoms with deuterium and investigate the impact that isotopic substitution has on the dynamics at the active site by 2D IR spectroscopy and compare to measurements of the temperature dependence of the intrinsic KIEs. We show that ^{15}N labeling of FDH has the largest effect and makes the active site more heterogeneous, while the addition of non-exchangeable deuterium appears to have the opposite effect of ^{15}N on active site dynamics, resulting in behavior similar to the native FDH. Nevertheless, the temperature dependence of the KIE shows a monotonic trend with protein mass that does not correspond with the changes in dynamics. These results suggest that isotope labeling has more than just a mass effect on enzyme dynamics and may influence electrostatics in ways that complicate the interpretation of the protein isotope effect.

INTRODUCTION

Enzymes are dynamic systems with motions that occur over a broad range of length and timescales. Binding or release of substrates and products are often the slowest steps in an enzyme-catalyzed reaction and often determine the steady state rate constants (i.e. k_{cat}).[1] The faster motions (fs-ps time-scale) of the enzyme that can be relevant to bond cleavage[2, 3] are harder to probe experimentally because the transition state of an enzyme-catalyzed reaction is short-lived and few experimental methods offer the required time resolution for such measurements. Thus, unraveling the dynamics at the transition state requires a clever approach.

One experimental approach for accessing information about the thermal sampling of conformations involved in the catalyzed reaction is the temperature dependence of primary kinetic isotope effects (KIEs).[4, 5] Temperature independent KIEs have been interpreted to reveal an active-site structure that minimizes both the average donor-acceptor distance (DAD) and the distribution of distances between the substrates. Correspondingly, an increase in the temperature dependence of the KIE for these enzymes indicates an active-site structure that requires thermally activated sampling of a broad DAD distribution.[6] The temperature dependence of KIEs for active-site mutants probes local perturbations of the active site structure (shortening or broadening of the distance between donor and acceptor atoms),[7-9] while the effects of remote mutation [10, 11] (~8-10 Å or more away from the active site) probes the effects of networks of coupled residues on the catalyzed reaction. Recently, isotopic substitution of the protein[12-16] has been used to perturb the time scales of structural fluctuations of the enzyme that participate in the catalyzed reaction. The extent to which this perturbation is strictly a mass effect on the dynamics, however, has been hotly debated.[17, 18] Two-dimensional infrared (2D IR) spectroscopy is an excellent tool for directly measuring protein dynamics at timescales from hundreds of femtoseconds to tens of picoseconds and has enabled the investigation of the active-site dynamics in the enzyme formate dehydrogenase (FDH).[19, 20] FDH is a unique and convenient model system that is amenable to a combination of KIEs and vibrational spectroscopic techniques for elucidating the relationship between hydride transfer and the protein dynamics.[19] Figure 1 shows the crystal structure of FDH (PDB 5DN9),[21] which is a homodimer with two independent active sites. The inset shows a close-up view of one active site of the ternary complex with NAD^+ that illustrates the hydrophobic packing of the active site and the close positioning of the azide with respect to the nicotinamide ring. FDH catalyzes a hydride ion transfer from formate ion to NAD^+ forming CO_2 and NADH. The azide anion (N_3^-) is a competitive inhibitor for the enzyme that mimics the transition state of the reaction catalyzed by FDH.[22] Here we report 2D IR measurements of the azide anion bound to isotopologues of FDH to investigate the effect of isotopic substitution on FDH structure and dynamics.

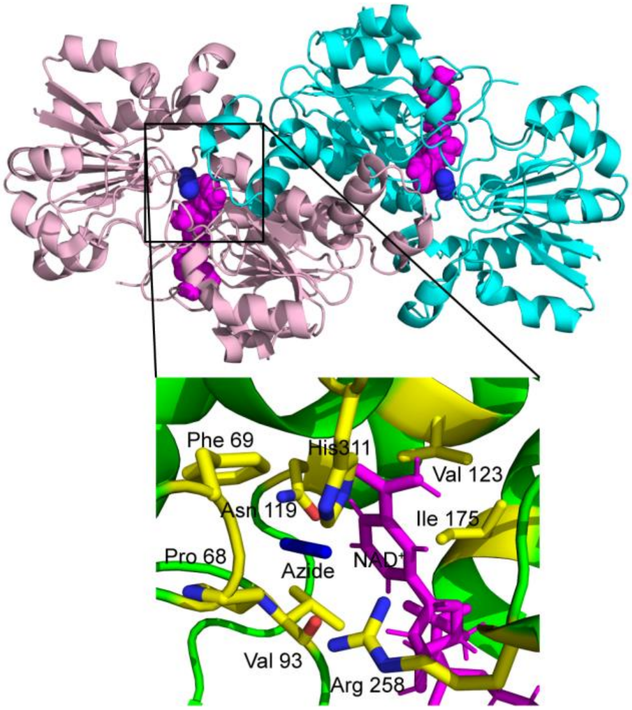


Figure 1: Crystal structure of the FDH homodimer (PDB 5DN9) with azide shown in blue, NAD^+ in magenta, and each of the two protein chains shown in pink and cyan. Inset shows a zoomed in view of the active site labeling key residues along with the cofactor and azide anion.

We have previously reported kinetic measurements on hydride transfer catalyzed by differently labeled FDH isotopologues.[18] The working theory in that work was that isotopic substitution of atoms to their heavier isotopes i.e. ^{13}C, ^{15}N, and nonexchangeable hydrogen with deuterium (^{2}H) only increased the mass of the enzyme, diminishing the frequencies of motions that directly affect the reactive complex, and did not alter the electrostatics of the enzyme.[16] As a result, we anticipated seeing an effect on the hydride transfer step of the reaction without any impact on steady state parameters (k_{cat} and K_m) that are largely influenced by electrostatic interactions between the enzyme and substrates. These studies compared kinetics and KIEs for ^{13}C-^{15}N (doubly labeled), ^{13}C-^{15}N-^{2}H (triply labeled) and native (unlabeled) FDH. As discussed in ref [18], we observed an increase in the temperature dependence of the KIEs that was inversely proportional to the square root of the mass, i.e. the slope of the KIE plot $\propto \sqrt{\frac{1}{protein\ mass}}$, suggesting that there was a relationship between vibrational motions of the enzyme and the kinetics of the hydride transfer step. Nevertheless, the steady state kinetic experiments revealed that k_{cat} and K_m values were altered in a way that was not mass dependent. The changes in K_m reflected that ligand binding was altered, suggesting isotopic changes to the electrostatic interactions in FDH.[18] Although the conclusion of this study was that the isotope labeling was primarily a mass effect on the hydride transfer, the steady-state kinetic results suggested that the situation may be more complicated than what could be inferred from these measurements alone.

In the present study, we use 2D IR to probe the protein dynamics in the active site directly and to determine the effects of protein isotopic labeling on those dynamics. In addition to the ^{13}C-^{15}N and ^{13}C-^{15}N-^{2}H labeled FDHs, we also report kinetic and spectroscopic measurements of ^{15}N labeled FDH. Surprisingly, we find that uniform labeling of FDH with ^{15}N causes the largest change to the observed dynamics by increasing the amplitude of the long-time decay component of the azide frequency-frequency correlation function (FFCF) and slightly accelerating the rate of that decay. These observations indicate that the enzyme active site becomes more heterogeneous as a result of this isotopic substitution. By comparison, ^{13}C substitution appears to have a negligible effect on FDH structure and dynamics. Interestingly, introduction of all three isotopes (^{13}C, ^{15}N, and ^{2}H) together restores the measured dynamics to those of the native enzyme. These observations show that isotopic substitution has more than just a mass effect and that even for modest substitutions, such as ^{15}N, the electrostatic perturbation on the structure and dynamics can be significant.

MATERIALS AND METHODS

Preparation of FDH isotopologues. FDH preparation follows previously published methods.[18] Briefly, labeled FDHs were expressed in BL21 (DE3) cells deficient in the rne131 gene (Invitrogen) in standard M9 minimal media. M9 media prepared in H_2O (for ^{15}N and ^{13}C-^{15}N FDH) or 99.8% D_2O (for ^{13}C-^{15}N-^{2}H FDH), was supplemented with minimal essential medium vitamins (Thermo Fisher), and $^{15}NH_4Cl$ and D-glucose (U-$^{13}C_6$, 99% for ^{13}C-^{15}N FDH or U-$^{13}C_6$, 99%; 1, 2, 3, 4, 5, 6, 6-$^{2}H_7$, 97-98% for ^{13}C-^{15}N-^{2}H FDH) were used as the sole nitrogen and carbon sources, respectively. All enzymes were purified to homogeneity in buffered H_2O using affinity chromatography according to methods described elsewhere.[18, 21, 23, 24] The different FDH isotopologues exhibit monomeric molecular weights of 40.31, 40.76, 42.54 and 44.78 kDa for unlabeled, ^{15}N, ^{13}C-^{15}N and ^{13}C-^{15}N-^{2}H FDH, respectively, as determined by ESI-MS (Figure S1). Thus, the masses of ^{15}N, ^{13}C-^{15}N and ^{13}C-^{15}N-^{2}H FDHs are 1.1%, 5.5% and 11.1% heavier than the unlabeled enzyme, respectively.

2D IR spectroscopy. The apparatus and sample preparation for 2D IR measurements has been described previously.[20, 25] Optical parametric amplification in β-barium borate (BBO) followed by difference frequency generation in $AgGaS_2$ produces ~120 fs mid-IR pulses centered at 2050 cm^{-1} that are separated into pump and a probe pulses. A pulse shaper further separates the pump pulse into two pulses separated in time by τ. A computer-controlled translation stage determines the waiting time, T, between the second pump pulse and the probe pulse. For each

2D IR spectrum, a cosine windowing function apodizes τ to 4 ps. Following up-conversion to the visible region of the spectrum, a spectrometer disperses the probe spectrum for detection by a 1024-pixel visible array detector, which gives the spectral response along the ω_{probe} axis. We use a 4-pulse phase cycle and calculate the change in the probe absorbance caused by sequential interactions with the first two pump pulses at each time delay to give a purely absorptive 2D IR signal. Fourier transformation with respect to τ gives the ω_{pump} axis. Waiting times range from 0 to 5 ps, in 50 fs steps.

2D IR sample preparation. The FDH-NAD$^+$-N_3^- ternary complex is prepared in 100 mM phosphate buffer at pH 7.5. The final concentrations for FDH, NAD$^+$ and azide are 1.6, 2 and 1.5 mM, respectively. A 5 µL sample of the ternary complex is placed between CaF_2 windows with a 50 µm spacer in a temperature-controlled cell (Harrick Scientific) and incubated at 5°C for both 2D IR and FT IR measurements.

Data analysis. The centerline slope (CLS) analysis method[26, 27] is used to quantify changes in the 2D IR line shapes. A plot of the CLS vs waiting time gives information about the loss of frequency correlations for the azide anion in the FDH active site environment. To extract the FFCF from 2D IR spectra we use an approach that has been described in detail previously.[20] In summary, we fit the CLS data to the minimum number of exponential decay terms including oscillatory contributions as necessary. In this case we use a sum of two oscillating exponentials and a decaying exponential:

$$F(\mathrm{T}) = A_0 \mathrm{e}^{-\frac{\mathrm{T}}{\tau_0}} + \sum_{\mathrm{i}} A_i \mathrm{e}^{-\frac{\mathrm{T}}{\tau_{\mathrm{i}}}} * \cos(2\pi\omega_{\mathrm{i}}\mathrm{T} + \phi_{\mathrm{i}}) \quad (1)$$

Where the τ_i values reflect the time scales for the decay of the correlations, ω_i are the frequencies of the underdamped oscillations, and the A_i values are the relative amplitudes from the fit to the

CLS. The absolute amplitude of each corresponding component of the FFCF, Δ_i, can then estimated from the FWHM of the FTIR spectrum using the method provided by Kwak, et al.[26]

$$\Delta_i = \sqrt{\frac{A_i}{\sum_i A_i}} \frac{FWHM}{2\sqrt{2ln2}} \tag{2}$$

The dephasing time, T_2, is also estimated from the FWHM of the FTIR spectrum:

$$T_2 = \frac{1}{\pi * FWHM * \sqrt{\sum_i A_i}} \tag{3}$$

Measurement of Intrinsic KIEs. The competitive H/T and D/T KIEs (H- hydrogen, D- deuterium and T- tritium) for the second order rate constant (k_{cat}/K_m) of the hydride transfer reaction of FDH follow well established methods published elsewhere.[18, 21] The temperature range is 5-45 ˚C. Intrinsic KIEs for the FDH isotopologues are determined from H/T and D/T observed KIEs using the Northrop equation[18, 28, 29] (eq 4):

$$\frac{{}^{T}(V/K)_H^{-1} - 1}{{}^{T}(V/K)_D^{-1} - 1} = \frac{({}^{T}k_{hyd})^{-1} - 1}{({}^{T}k_{hyd})^{-1/3.34} - 1} \tag{4}$$

where ${}^{T}k_{hyd}$ is the intrinsic H/T KIE, ${}^{T}(V/K)_H$ and ${}^{T}(V/K)_D$ are observed H/T and D/T KIEs respectively. Isotope effects on the activation parameters are calculated by a non-linear fit of the data to the Arrhenius equation (eq 5):

$$KIE = \frac{k_{light}}{k_{heavy}} = \frac{A_{light}}{A_{heavy}} \exp(\frac{\Delta E_{a(heavy-light)}}{RT}) \tag{5}$$

where k_{light} and k_{heavy} are rate constants for light (^{1}H) and heavy (^{3}H) substrate isotopologues respectively, A_{light}/A_{heavy} is the isotope effect on the Arrhenius pre-exponential factor, $\Delta E_{a(heavy-light)}$ which is equivalent to $-\Delta E_{a(light-heavy)}$,is the difference in activation free energies between the heavy and light substrate isotopologues given by the slope of the Arrhenius plot, *R* is the universal gas constant and *T* is absolute temperature.

Circular Dichroism (CD) Spectroscopy and Melting Temperature Measurements.

CD spectra for thermal denaturation of $_{15}$N FDH are measured for a temperature range of 25-75°C, across wavelengths ranging from 190 to 240 nm, following previously published methods.[30] The spectra are compared and contrasted with those of the other FDH isotopologues, namely, light FDH, $_{13}$C-$_{15}$N FDH and $_{13}$C-$_{15}$N-$_{2}$H FDH.[18] Enthalpy (ΔH) and entropy (ΔS) of folding and change in heat capacity between folded and unfolded states (ΔC_p) are determined for the four FDH isotopologues following previously published methods.[31, 32] The mid-points of thermal denaturation (here defined as T_m) are calculated by fitting the data to a five-parameter sigmoidal curve of the form, θ (millidegrees) = y_0+a/(1+exp(-(T-T_m)/b))^c, where θ is ellipticity. Mean residue ellipticities are collected at 222 nm for all the FDH isotopologues across the stated temperature range with 1°C increments. Ellipticity at 222 nm corresponds to a signature peak of alpha helix structure, and the secondary structure of *Cb*FDH contains more than 45% alpha helix character. Monomeric concentrations of the protein samples are, 0.48, 0.48. 0.28 and 0.25 mg/mL, for light, $_{15}$N, $_{13}$C-$_{15}$N and $_{13}$C-$_{15}$N-$_{2}$H FDHs, respectively, and they are prepared in 100 mM phosphate buffer at pH 7.5. The similarity among the CD spectra (Figure S2) suggests that the secondary structures of the FDH isotopologues are not substantially perturbed by the isotopic labeling.

RESULTS AND DISCUSSION

Protein Electrostatic Perturbations Revealed by 2D IR and Melting Temperature Measurements.

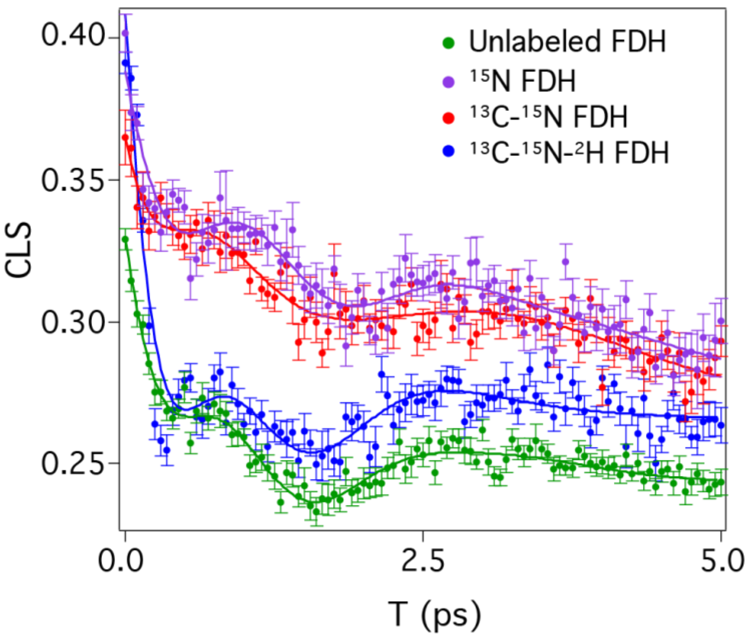


Figure 2: Centerline slope decays for azide bound to FDH isotopologues. The points are the average of 20-25 trials with error bars that are the standard error of the mean, and the solid lines are the fits to eq 1. Data for the unlabeled enzyme are previously published results from ref 20.

Figure 2 shows the CLS decays for azide bound to isotopologues of FDH with various labeling combinations, along with the previously published data for the native enzyme.[20] As with the native enzyme, the CLS decay for all of the labeling schemes are fit to two oscillating exponentials that decay on the picosecond and sub-picosecond timescales, and a longer exponential decay that persists for tens of picoseconds. This longer decay component is sometimes referred to as a "static" component, especially when the slow time constant, τ_0, is fixed to infinity ($\tau_0 = \infty$). In that case, this term only reflects the amplitude of the static offset of the FFCF, which occurs when the FFCF decay is much slower than the lifetime of the chromophore such that frequency distribution is not fully sampled within the timescale of the 2D IR measurement. In the data in Figure 1, fixing this value to infinity gives poor fits to the data and does not account for the overall negative going slope of the long-time CLS decay. Therefore, we let τ_0 be a fit parameter when fitting the CLS decay and refer to it as a pseudo-static component as it persists for at least an order of magnitude longer than azide's vibrational lifetime allows us to measure. As a result of the long time-scale of this decay compared to the lifetime, the uncertainty in the actual decay time for these fits is quite large. This pseudo-static component appears to be the one most affected by isotopic substitution of heavy atoms in FDH. Table 1 lists all the fit parameters for each isotopologue of FDH.

Table 1: FFCF parameters for azide bound to isotopologues of FDH. Reported uncertainties come from fits to the data weighted by the standard error of the means for each data point. [a]from ref 20

FDH	Δ_0 (cm^{-1})	τ_0 (ps)	Δ_1 (cm^{-1})	τ_1 (ps)	ω_1 (cm^{-1})	Δ_2 (cm^{-1})	τ_2 (ps)	ω_2 (cm^{-1})	T_2 (ps)
Unlabeled[a]	1.54± 0.04	80 ± 40	0.66± 0.05	0.64 ± 0.19	24 ± 1	0.79 ± 0.03	1.08 ± 0.13	9.9 ± 0.4	2.4 ± 0.4
^{15}N	1.76 ±0.05	29 ± 10	0.65 ± 0.06	0.83± 0.35	20 ± 2	0.66 ± 0.03	1.24 ±0.38	9.4 ± 0.8	2.3 ± 0.4
^{13}C-^{15}N	1.73 ±0.07	30 ± 10	0.61 ± 0.14	0.41± 0.34	22 ± 6	0.55 ± 0.04	1.73 ±0.64	9.1 ± 0.6	2.3 ± 0.9
^{13}C-^{15}N-^{2}H	1.59 ±0.05	130 ± 80	1.10 ± 0.06	0.51 ± 0.09	21 ± 2	1.09 ± 0.03	0.84 ±0.10	10.8 ± 0.6	2.0 ± 0.4

Surprisingly, enrichment of the protein with ^{15}N causes the largest change in the CLS decay compared to the native enzyme. For this isotopologue, the amplitude of the pseudo-static component (Δ_0) increases and the time constant of this term (τ_0) decreases. In contrast, most of the other CLS decay parameters do not change significantly. Enrichment of FDH with both ^{15}N and ^{13}C has nearly the same effect as the ^{15}N substitution alone. By deduction, this suggests that the effect of exchanging ^{14}N to ^{15}N is significant but that the effect of exchanging ^{12}C to ^{13}C is not and that incorporation of ^{13}C alone would have little to no effect on the enzyme dynamics. This result indicates that the effect is not just a mass effect given that the change in mass as a result of ^{15}N substitution is much less than that for ^{13}C substitution. Finally, replacement of non-exchangeable ^{1}H to ^{2}H appears to have the opposite, almost fully compensatory, effect on the amplitude of the FFCF compared with ^{15}N, as incorporation of all three isotopes (^{15}N, ^{13}C, and ^{2}H) into FDH results in a value for Δ_0 that is nearly identical to that for native, natural abundance

FDH (Table 1). Interestingly, the triply labeled FDH is also the only isotopologue that significantly affects the oscillatory contributions to the FFCF, as amplitudes of both of the oscillatory terms (Δ_1 and Δ_2) increase for this isotopologue compared to the unlabeled enezyme. For none of the isotopologues, however, is there a significant change in the frequencies of the oscillatory components of the FFCF as a result of the mass change.

Broadly speaking, the motions of FDH probed by the azide can be placed into two categories. First there are the oscillatory contributions. These decay over relatively short timescales and likely involve primarily local motions of the nicotinamide ring and the immediate active-site residues of FDH.[20] All of the other dynamics are reflected in the long-time decay or pseudo-static component. These longer time scale motions likely extend well beyond the active site, and could involve motions of residues across the entire protein. Deuterium labeling of FDH apparently has an effect that offsets the perturbation introduced by ^{15}N in the triply labeled protein. We know that the C-H bond is longer than the C-D bond due to anharmonicity and zero-point effects. Therefore, the C-H has a slightly larger bond dipole than the C-D bond.[33] Consequently, C-D bonds, particularly in aliphatic regions of the enzyme, are more hydrophobic than the corresponding C-H bonds, which may increase the stability of hydrophobic regions of the enzyme. Similarly, for the influence of ^{15}N labeling, the bond length of the ^{15}N-H bond will be somewhat less than that for ^{14}N-H leading to smaller bond dipoles and weaker hydrogen bonds. Weakening the hydrogen bonds will destabilize the overall protein fold making the enzyme more flexible and heterogeneous, overall, consistent with the spectroscopic measurements. These results are in stark contrast to the notion that isotopic substitution creates a "Born-Oppenheimer-like"[16] enzyme that perturbs only the mass and not the electrostatics of the enzyme.

Thermal denaturation measurements provide further evidence of the structural impacts of isotopic substitutions. In our previous study, we reported melting temperature measurements (T_m) of the native, doubly labeled (^{13}C-^{15}N) and triply labeled (^{13}C-^{15}N-^{2}H) FDH isotopologues.[18] Because the triply labeled and native enzymes showed nearly identical T_m values and the doubly labeled melting temperature was depressed by only 2.0°C from the native enzyme, we concluded that this modest decrease in T_m for the doubly labeled enzyme was insignificant. There appeared to be no trend with mass, and, at most, we suggested that there may be some modest differences in electrostatics that were responsible for the change in T_m. Adding the T_m value for ^{15}N labeled FDH along with the previously measured values, however, reveals a pattern consistent with that seen in the 2D IR experiments (Figure 3 and Table 2). Table 2 lists the enthalpy change of folding (ΔH), entropy change of folding (ΔS), and the change in heat capacity between folded and unfolded states (ΔC_p). Consistent with the 2D IR results, ^{15}N labeling yields the lowest enthalpy of folding, and the . Together these results show that the changes in protein heterogeneity and flexibility observed by 2D IR measurements, reflect changes in the overall protein stability. T_m measurements, when considered together with 2D IR experiments provide solid evidence that the ^{15}N and ^{2}H substitutions significantly impact protein electrostatics.

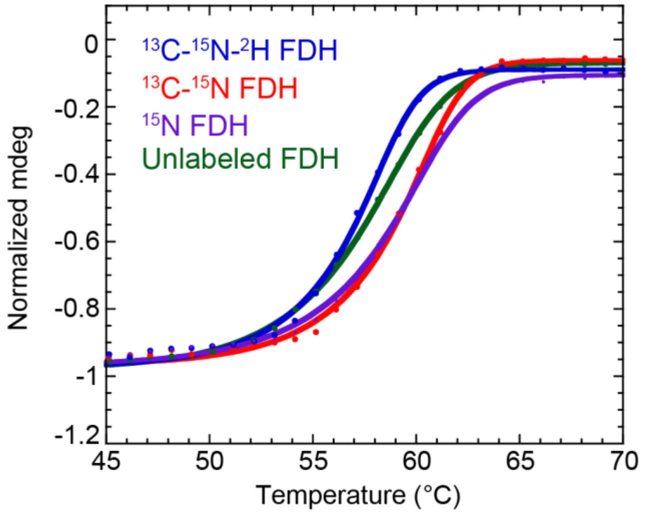


Figure 3: Thermal denaturation data (points) and fits for FDH and its isotopologues

Table 2: Enthalpy change of folding (ΔH), entropy change of folding (ΔS), change in heat capacity between folded and unfolded states (ΔC_p), and melting temperatures (T_m) for different FDH isotopologues, calculated using equations given in ref [31]. [a]from ref 18.

FDH	Light	^{15}N	^{13}C-^{15}N	^{13}C-^{15}N-^{2}H
ΔH (kcal/mol)	-111.8 ± 2.2	-103.8 ± 4.0	-122.9 ± 4.3	-130.4 ± 3.8
ΔS (kcal/mol.K)	-0.336 ± 0.007	-0.310 ± 0.012	-0.367 ± 0.013	-0.393 ± 0.012
ΔC_p (kcal/mol.K)	-4.7 ± 0.9	-4.0 ± 2.0	-5.0 ± 2.0	-5.8 ± 1.5
T_m (˚C)	59.5 ± 0.2 [a]	61.5 ± 0.1	61.5 ± 0.3 [a]	59.0 ± 0.2[a]

Isotopic Labeling Effects on Bond Vibrational Motions as Revealed by Temperature Dependence of KIEs.

Figure 4 shows a plot of the temperature dependence of the intrinsic KIEs (left). The slope of these Arrhenius curves gives the isotope effect on the activation energy, which we report as $\Delta E_{a(T\text{-}H)} = E_{a(T)} - E_{a(H)}$. Figure 4 (right) shows the correlation of the change in activation energy from the KIE measurements versus the reciprocal of the square root of protein mass for the FDH isotopologues. Though the overall trend of the temperature dependence of the KIEs varies monotonically, the correlation between the temperature dependence of the KIE and the square root of the reciprocal of the protein mass is distinctly nonlinear.

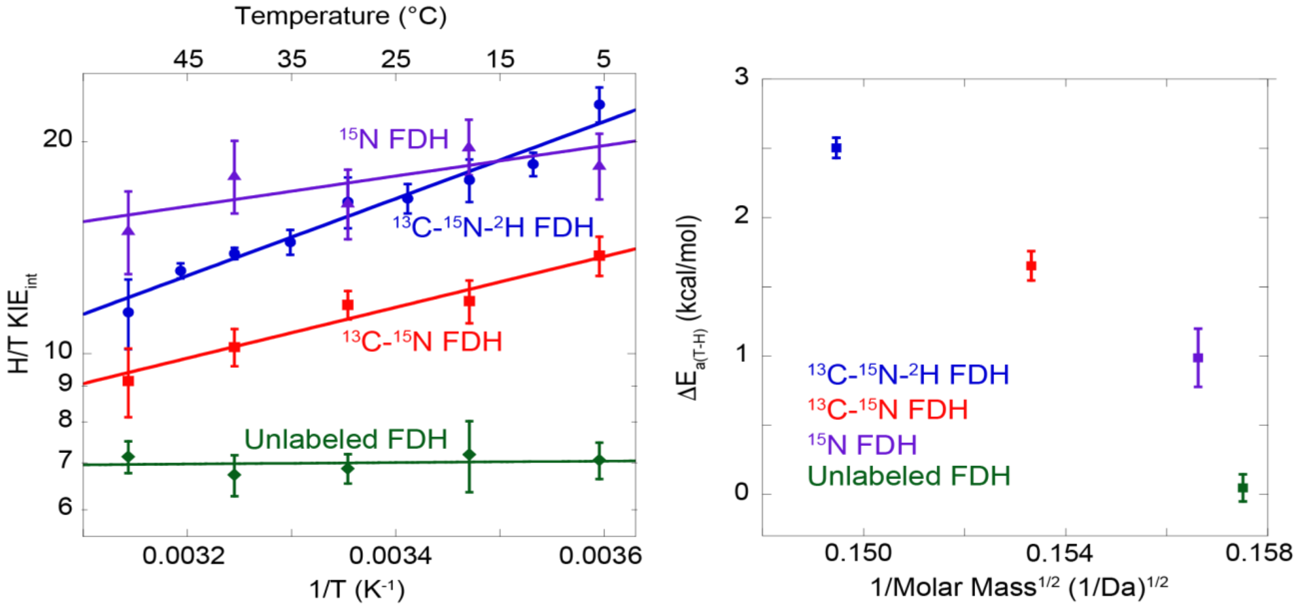


Figure 4: H/T intrinsic KIEs (left) for native FDH[a], ^{15}N labeled FDH, ^{13}C;^{15}N labeled FDH[a] and ^{13}C;^{15}N;^{2}H labeled FDH[a] as a function of reciprocal temperature. Points are the means and uncertainties are the standard deviations for ~16 intrinsic KIE values resulting from a set of 4 H/T and 4 D/T observed KIE values at each temperature. Activation energy differences (right) between the two substrate isotopologues (slopes of the Arrhenius plots) as a function of reciprocal of the square root of protein mass. Error bars are the parameter uncertainties from fits to the Arrhenius plots performed as nonlinear fits to the unlinearized data. [a]from reference 18.

Interestingly, the magnitudes of the KIEs do not follow a mass trend at all. In the phenomenological activated tunneling models used to interpret these KIE experiments, the overall magnitude of the KIE is indicative of the average DAD at the TRS of hydride transfer for those hydride nuclei capable of tunneling.[2] Typically, the wild-type enzyme has a relatively small isotope effect, and perturbations such as mutagenesis,[7, 34] isotope labeling,[12, 18] or pressure changes,[35] for example, tend to disturb the precise packing of the active-site. A broader distribution of DADs, increases the temperature dependence of the KIE, and a distribution centered at longer average DAD inflates the overall magnitude of the KIE.[6] Somewhat surprisingly, the highest KIEs are seen for the ^{15}N labeled FDH suggesting that this isotopologue has a more distorted active-site conformation as a result of isotopic substitution. The increase in

KIE values in ^{15}N FDH suggests that the electrostatic perturbations in the protein ensemble contribute to the observed change in the KIE and its temperature. In fact, the temperature dependences of both the ^{15}N labeled and the ^{13}C-^{15}N labeled proteins are inflated relative to what would be expected based on a mass effect alone. The magnitude of the KIE for the doubly labeled protein is less than that for the singly labeled ^{15}N FDH. There is little effect of the ^{13}C substitution seen in either the T_m or the 2D IR results. Nevertheless, the overall magnitude of the isotope effect decreases and the slope increases for the doubly labeled protein relative to those values for the ^{15}N enzyme, suggesting that there is also some effect on the KIE that is not reflected in either the 2D IR or the melting temperature. These results indicate that the effects of isotopic substitution on the KIE for the hydride transfer are far more complex than a mere mass effect, and they defy any simplistic interpretations. Thus, correlations of the KIEs with the square root of the reciprocal mass for a series of protein isotopologues should be interpreted with caution.

Because the measured active-site dynamics do not change in a way that is correlated with the protein mass, it is clear that the changes in the KIE should not be interpreted in terms of just a mass effect. Furthermore, the fact that ^{15}N and ^{2}H substitutions have the largest effects on the dynamics and in opposite directions suggests that the trend in the temperature dependence of the KIE with the reciprocal of the square root of the protein mass could be fortuitous. Though, in principle, computational methods could be helpful in understanding these phenomena,[36] if electrostatics are perturbed significantly (as these experiments suggest), then the molecular dynamics force fields for simulations would not be suitable for capturing the effects of isotopic substitutions on the physical phenomena we report without reparametrizing the force field for each isotopologue. In addition, these results also raise questions about perturbations to the protein structure and dynamics introduced by isotopic labeling for NMR measurements. Isotopic labeling is the crux of NMR spectroscopy. However, there is limited information in the literature about the isotopic labeling effects on overall protein structure and stability with respect to NMR studies. The most relevant prior literature discusses the effects of deuteration[37] of the proteins or D_2O solvent isotope effects[38] and suggests that isotopic labeling primarily affects hydrogen bonding. We show that uniform substitution with ^{15}N, which is very common in NMR measurements and widely believed to be nonperturbative, has significant effects on the long-time dynamics of the protein and the temperature at which the protein unfolds. These results warrant further study to better understand the effects of isotopic labeling on protein structure and dynamics as they relate to a large body of work where NMR spectroscopy is employed. This could be an interesting field of study in general if the results for FDH are relevant for other systems as well.

CONCLUSIONS

This study reports the effects of isotopic substitution on the dynamics of FDH and the temperature dependence of KIEs. We show that incorporation of ^{15}N impacts the magnitude and time scale of the long-time decay component of the FFCF of azide bound to FDH, indicating that azide experiences larger amplitude frequency fluctuations that decay more quickly, and that the oscillatory features of azide's FFCF remain mostly unchanged. In contrast, ^{13}C has little impact on the observed dynamics. Deuterium, however, appears to have a compensatory effect on the dynamics compared to ^{15}N, restoring the behavior of the triply labeled isotopologue (^{13}C-^{15}N-^{2}H FDH) to that of the native FDH. These observations, together with the melting temperature results, suggest that isotope labeling of the enzyme impacts more than just the mass of the enzyme, and perturbs electrostatics in a way that complicates the interpretation of the KIE experiments on the series of isotopically labeled proteins.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge funding for this work from the National Science Foundation under grant CHE-1707598 (CMC).

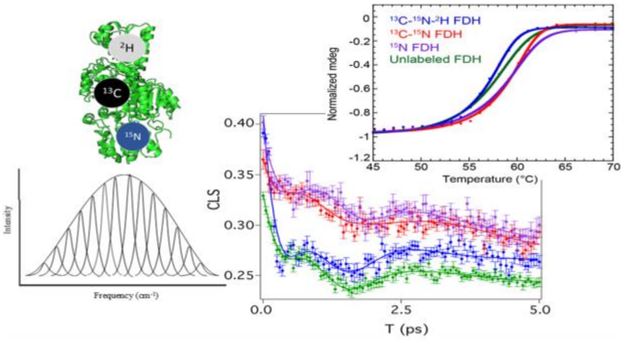
REFERENCES

1. Agarwal, P. K., Enzymes: An Integrated View of Structure, Dynamics and Function. *Microb. Cell Fact.* **2006,** *5* (1), 2.
2. Kohen, A., Role of Dynamics in Enzyme Catalysis: Substantial vs. Semantic Controversies. *Acc. Chem. Res.* **2015,** *48* (2), 466-473.
3. Schramm, V. L.; Schwartz, S. D., Promoting Vibrations and the Function of Enzymes. Emerging Theoretical and Experimental Convergence. *Biochemistry* **2018,** *57* (24), 3299-3308.
4. Klinman, J. P., An Integrated Model for Enzyme Catalysis Emerges from Studies of Hydrogen Tunneling. *Chem. Phys. Lett.* **2009,** *471* (4-6), 179-193.
5. Klinman, J. P.; Kohen, A., Hydrogen Tunneling Links Protein Dynamics to Enzyme Catalysis. *Annu. Rev. Biochem.* **2013,** *82* (1), 471-496.
6. Roston, D.; Islam, Z.; Kohen, A., Isotope Effects as probe for Enzyme Catalyzed Hydrogen-Transfer Reactions. *Molecules* **2013,** *18*, 5543-5567.
7. Stojkovic, V.; Perissinotti, L. L.; Willmer, D.; Benkovic, S. J.; Kohen, A., Effects of the Donor-Acceptor Distance and Dynamics on Hydride Tunneling in the Dihydrofolate Reductase Catalyzed Reaction. *J. Am. Chem. Soc.* **2012,** *134* (3), 1738-45.
8. Bahnson, B. J.; Colby, T. D.; Chin, J. K.; Goldstein, B. M.; Klinman, J. P., A Link between Protein Structure and Enzyme Catalyzed Hydrogen Tunneling. *Proc. Natl. Acad. Sci. U. S. A.* **1997,** *94* (24), 12797-12802.
9. Colby, T. D.; Bahnson, B. J.; Chin, J. K.; Klinman, J. P.; Goldstein, B. M., Active Site Modifications in a Double Mutant of Liver Alcohol Dehydrogenase: Structural Studies of Two Enzyme-Ligand Complexes. *Biochemistry* **1998,** *37* (26), 9295-304.
10. Wang, Z.; Abeysinghe, T.; Finer-Moore, J. S.; Stroud, R. M.; Kohen, A., A Remote Mutation Affects the Hydride Transfer by Disrupting Concerted Protein Motions in Thymidylate Synthase. *J. Am. Chem. Soc.* **2012,** *134* (42), 17722-30.
11. Singh, P.; Sen, A.; Francis, K.; Kohen, A., Extension and Limits of the Network of Coupled Motions Correlated to Hydride Transfer in Dihydrofolate Reductase. *J. Am. Chem. Soc.* **2014,** *136* (6), 2575-82.
12. Wang, Z.; Singh, P.; Czekster, C. M.; Kohen, A.; Schramm, V. L., Protein Mass-Modulated Effects in the Catalytic Mechanism of Dihydrofolate Reductase: Beyond Promoting Vibrations. *J. Am. Chem. Soc.* **2014,** *136* (23), 8333-41.
13. Kipp, D. R.; Silva, R. G.; Schramm, V. L., Mass-Dependent Bond Vibrational Dynamics Influence Catalysis by HIV-1 Protease. *J. Am. Chem. Soc.* **2011,** *133* (48), 19358-61.
14. Antoniou, D.; Ge, X.; Schramm, V. L.; Schwartz, S. D., Mass Modulation of Protein Dynamics Associated with Barrier Crossing in Purine Nucleoside Phosphorylase. *J. Phys. Chem. Lett.* **2012,** *3* (23), 3538-3544.
15. Francis, K.; Sapienza, P. J.; Lee, A. L.; Kohen, A., The Effect of Protein Mass Modulation on Human Dihydrofolate Reductase. *Biochemistry* **2016,** *55* (7), 1100-6.
16. Silva, R. G.; Murkin, A. S.; Schramm, V. L., Femtosecond Dynamics Coupled to Chemical Barrier Crossing in a Born-Oppenheimer Enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **2011,** *108* (46), 18661-5.
17. Kholodar, S. A.; Ghosh, A. K.; Kohen, A., Chapter Three - Measurement of Enzyme Isotope Effects. In *Methods in Enzymology*, Harris, M. E.; Anderson, V. E., Eds. Academic Press: 2017; Vol. 596, pp 43-83.

18. Ranasinghe, C.; Guo, Q.; Sapienza, P. J.; Lee, A. L.; Quinn, D. M.; Cheatum, C. M.; Kohen, A., Protein Mass Effects on Formate Dehydrogenase. *J. Am. Chem. Soc.* **2017,** *139* (48), 17405-17413.
19. Cheatum, C. M.; Kohen, A., Relationship of Femtosecond-Picosecond Dynamics to Enzyme-Catalyzed H-Transfer. *Top Curr. Chem.* **2013,** *337* (Dynamics in Enzyme Catalysis), 1-39.
20. Pagano, P.; Guo, Q.; Kohen, A.; Cheatum, C. M., Oscillatory Enzyme Dynamics Revealed by Two-Dimensional Infrared Spectroscopy. *J. Phys. Chem. Lett.* **2016,** *7* (13), 2507-11.
21. Guo, Q.; Gakhar, L.; Wickersham, K.; Francis, K.; Vardi-Kilshtain, A.; Major, D. T.; Cheatum, C. M.; Kohen, A., Structural and Kinetic Studies of Formate Dehydrogenase from Candida boidinii. *Biochemistry* **2016,** *55* (19), 2760-71.
22. Blanchard, J. S.; Cleland, W. W., Kinetic and Chemical Mechanisms of Yeast Formate Dehydrogenase. *Biochemistry* **1980,** *19* (15), 3543-3550.
23. Labrou, N. E.; Karagouni, A.; Clonis, Y. D., Biomimetic-Dye Affinity Adsorbents for Enzyme Purification: Application to the One-Step Purification of Candida boidinii Formate Dehydrogenase. *Biotechnology and bioengineering* **1995,** *48* (3), 278-88.
24. Labrou, N. E., Improved Purification of Candida boidinii Formate Dehydrogenase. *Bioseparation* **2000,** *9* (2), 99-104.
25. Rock, W.; Li, Y. L.; Pagano, P.; Cheatum, C. M., 2D IR spectroscopy using Four-Wave Mixing, Pulse Shaping, and IR Upconversion: A Quantitative Comparison. *J. Phys. Chem. A* **2013,** *117* (29), 6073-83.
26. Kwak, K.; Park, S.; Finkelstein, I. J.; Fayer, M. D., Frequency-Frequency Correlation Functions and Apodization in Two-Dimensional Infrared Vibrational Echo Spectroscopy: A New Approach. *J. Chem. Phys.* **2007,** *127* (12), 124503.
27. Guo, Q.; Pagano, P.; Li, Y.-L.; Kohen, A.; Cheatum, C. M., Line Shape Analysis of Two-Dimensional Infrared Spectra. *J. Chem. Phys.* **2015,** *142* (21), 212427/1-212427/13.
28. Northrop, D. B., Intrinsic Isotope Effects in Enzyme Catalyzed Reactions. In *Enzyme Mechanism from Isotope Effects*, Cook, P. F., Ed. CRC Press: Boca Raton, FL, 1991; pp 181-202.
29. Swain, C. G.; Stivers, E. C.; Reuwer, J. F.; Schaad, L. J., Use of Hydrogen Isotope Effects to Identify the Attacking Nucleophile in the Enolization of Ketones Catalyzed by Acetic Acid1-3. *J. Am. Chem. Soc.* **1958,** *80* (21), 5885-5893.
30. Galkin, A. G.; Kutsenko, A. S.; Bajulina, N. P.; Esipova, N. G.; Lamzin, V. S.; Mesentsev, A. V.; Shelukho, D. V.; Tikhonova, T. V.; Tishkov, V. I.; Ustinnikova, T. B.; Popov, V. O., Site-directed mutagenesis of the essential arginine of the formate dehydrogenase active centre. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2002,** *1594* (1), 136-149.
31. Greenfield, N. J., Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat Protoc* **2006,** *1* (6), 2527-2535.
32. Greenfield, N. J., Analysis of circular dichroism data. *Methods Enzymol.* **2004,** *383* (Numerical Computer Methods, Part D), 282-317.
33. Turowski, M.; Yamakawa, N.; Meller, J.; Kimata, K.; Ikegami, T.; Hosoya, K.; Tanaka, N.; Thornton, E. R., Deuterium Isotope Effects on Hydrophobic Interactions: The Importance of Dispersion Interactions in the Hydrophobic Phase. *J. Am. Chem. Soc.* **2003,** *125* (45), 13836-49.

34. Knapp, M. J.; Rickert, K.; Klinman, J. P., Temperature-Dependent Isotope Effects in Soybean Lipoxygenase-1: Correlating Hydrogen Tunneling with Protein Dynamics. *J. Am. Chem. Soc.* **2002,** *124* (15), 3865-3874.
35. Hay, S.; Johannissen, L. O.; Hothi, P.; Sutcliffe, M. J.; Scrutton, N. S., Pressure Effects on Enzyme-Catalyzed Quantum Tunneling Events Arise from Protein-Specific Structural and Dynamic Changes. *J. Am. Chem. Soc.* **2012,** *134* (23), 9749-9754.
36. Mincer, J. S.; Schwartz, S. D., Rate-Promoting Vibrations and Coupled Hydrogen-Electron Transfer Reactions in the Condensed Phase: A Model for Enzymatic Catalysis. *J. Chem. Phys.* **2004,** *120* (16), 7755-60.
37. Koruza, K.; Lafumat, B.; Vegvari, A.; Knecht, W.; Fisher, S. Z., Deuteration of Human Carbonic Anhydrase for Neutron Crystallography: Cell Culture Media, Protein Thermostability, and Crystallization Behavior. *Arch. Biochem. Biophys.* **2018,** *645*, 26-33.
38. Stadmiller, S. S.; Pielak, G. J., Enthalpic Stabilization of an SH3 Domain by D_2O. *Protein Sci.* **2018,** *27* (9), 1710-1716.

TOC Graphic


^{2}H
^{13}C
^{15}N
Intensity
Frequency (cm^{-1})
CLS
0.40
0.35
0.30
0.25
0.0
2.5
5.0
T (ps)
^{13}C-^{15}N-^{2}H FDH
^{13}C-^{15}N FDH
^{15}N FDH
Unlabeled FDH
Normalized mdeg
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
45
50
55
60
65
70
Temperature (°C)