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Oscillatory Enzyme Dynamics Revealed by Two-Dimensional Infrared Spectroscopy

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Abstract

Enzymes move on a variety of length and timescales. While much is known about large structural fluctuations which impact binding of the substrates and release of products, little is known about faster motions of enzymes and how these motions may influence enzyme catalyzed reactions. This letter reports frequency fluctuations of azide anion bound to the active site of formate dehydrogenase measured via 2D IR spectroscopy. These measurements reveal an underdamped oscillatory component to the frequency-frequency correlation function when the azide is bound to the NAD⁺ ternary complex. This oscillation disappears when the reduced cofactor is added, indicating that the oscillating contributions most likely come from the charged nicotinamide ring. These oscillatory motions may be relevant to donor-acceptor distance sampling of the catalyzed hydride transfer, and therefore may give future insights into the dynamic behavior involved in enzyme catalysis.

Graphical Abstract

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Supporting Information

Two supporting figures are found in the SI. The first figure shows 2D IR spectra for azide bound to both NAD⁺ and NADH ternary complexes at several waiting times, illustrating the time evolution of the lineshapes. The second figure shows the decay of the excited state absorption for azide bound to FDH and NAD⁺.

Keywords

Two-dimensional infrared spectroscopy; formate dehydrogenase; oscillation dynamics; protein dynamics

An interesting feature of many enzyme-catalyzed hydrogen-transfer reactions is that they exhibit temperature-dependent rates, but temperature-independent primary kinetic isotope effects (KIE) that can be made temperature dependent by perturbations of the enzymes such as active-site mutations.¹ These observations have motivated activated tunneling models^{2–3} in which motions of the heavy atoms of the enzyme bring the zero point energies of the transferring hydrogen in the reactant and product states to degeneracy where tunneling can occur. One interpretation of this result is that a temperature-independent KIE signifies a compact transition state structure that limits distance sampling between the H-donor and acceptor during bond activation. While this interpretation follows from the phenomenological model, direct experimental evidence for it is lacking.

Structural events that occur on the time scale of bond activation are challenging to probe. The first major obstacle is that the tunneling-ready state exists only for a brief instant of time, and it is not possible to measure it directly. Transition-state analogs, however, offer an opportunity for studying structural fluctuations of the enzyme in conformations relevant to the reactive complex. The next problem is that time-resolved spectroscopies require a chromophore that would not alter the structure and function of the active site, and would mimic the transition state under study. Formate dehydrogenase (FDH) from *Candida boidinii* offers a unique way to overcome both of these problems, as the azide anion (N_3^-) binds tightly in the active site in the place of the formate reactant as a transition-state-analog inhibitor. Because azide is negatively charged, like the formate reactant, and is isoelectronic with the CO_2 product, its binding creates a structural analog of the tunneling ready state for the enzyme-catalyzed reaction. In addition, the azide anion is a strong infrared chromophore that absorbs in an uncongested region of the mid-IR spectrum, providing a convenient

handle for probing the structural motions of the active site of FDH in a conformation that mimics the tunneling ready state.

Although we have explored this system previously by 3-pulse photon echo and 2D IR spectroscopies, previous measurements suffered from experimental shortcomings that adversely impacted the reported results.⁴⁻⁵ This letter serves as an important refinement of and correction to our previously published results. We report 2D IR measurements of the antisymmetric stretching vibration of the azide anion bound to FDH in ternary complexes with NAD⁺ and NADH with an improved 2D IR apparatus. Our measurements of the frequency-frequency correlation function (FFCF) for azide complexed to FDH with NAD⁺ show underdamped, oscillatory frequency fluctuations on the picosecond time scale, a result that was not seen in previous measurements and that suggests that the active-site insulates the reacting molecules from the thermal noise of the surrounding solvent. We show that these oscillations are likely related to motions of the nicotinamide ring of NAD⁺.

2D IR spectroscopy reveals the dynamics of the local environment around an ensemble of chromophores through the loss of frequency correlation as a function of the time between excitation and detection of the chromophores. At early waiting times, the local environment remains unchanged between excitation and detection. Thus, the probed frequency of each vibration correlates with that at which it was excited, giving rise to a peak elongated along the diagonal of the 2D IR spectrum. At increased waiting times, thermally activated motions lead to fluctuations of the environment around the azide and modulate its instantaneous vibrational frequency, leading to a loss of correlation between excitation and detection frequencies. This loss of correlation causes the 2D IR lineshape to become more circular. Thus, the timescales on which the lineshape changes directly report the timescales of the structural fluctuations of the environment. Figure 1 shows a representative 2D IR spectrum for azide in FDH complexed with NAD⁺ (For more spectra at different waiting times (T), see SI.) We use the centerline slope (CLS) analysis method⁶⁻⁷ to quantify changes in the 2D IR lineshapes. The purple circles in Figure 1 illustrate the centerline, and the white line is the linear fit to the centerline from which we get the slope. The centerline slope decay, CLS versus waiting time (T), is proportional to the FFCF of the azide anion.

Our previous measurements of azide anion bound to FDH ternary complexes via 3-pulse photon echo⁴ and four-wave mixing 2D IR⁵ spectroscopies suffered from a number of experimental shortcomings that affected the reported FFCF. First, homodyne detection of the 3-pulse photon echo signal meant that the signal amplitude decayed at twice the rate of population relaxation so that data could only be acquired out to 2 ps. Furthermore, these measurements suffered from a significant solvent background response near zero time delay, making it difficult to analyze the peak shift. The four-wave mixing 2D IR experiments had problems associated with phasing errors and apodization of the time-domain interferogram, which distort the lineshape, leading to errors in the extracted FFCF.⁷ In addition, the lineshape of azide bound to FDH is strongly motionally narrowed with a small spectral-diffusion contribution, which tends to depress the initial value of the CLS limiting the dynamic range of the measurement of the FFCF. These experimental features impacted the reported results in both of the previously published measurements in ways that we now understand and have worked hard to overcome.

Of these effects, the most significant involved the sampling methods for τ_1 in the four-wave mixing 2D IR experiment. In an effort to reduce data acquisition time, the 2D IR spectra in those measurements were collected with shorter apodization times at longer waiting times so that multiple replicates could be measured and averaged. Decreasing the apodization time, however, artificially deflates the value of the CLS.⁷ Because we systematically decreased the apodization time as we increased the waiting time we effectively forced the CLS to decay with increasing waiting time leading to the erroneous conclusion that the CLS for azide bound to FDH ternary complexes decays completely to zero. This conclusion would suggest that azide samples all configurations within our experimental window. Although the new results, measured with a consistent 4 ps apodization time, show a strongly motionally narrowed lineshape, it is also clear that there remains a non-negligible static offset in the FFCF at long waiting times.

Advances in our 2D IR apparatus using pulse shaping technology and infrared upconversion to visible wavelengths, have greatly improved both the signal-to-noise ratio and the phase stability of our measurements.⁸ The phase stability and rapid acquisition speed enable us to signal-average many, sometimes thousands, of 2D IR spectra to achieve excellent signal-to-noise ratios for the individual spectra. Furthermore, we measure and analyze the CLS values for many (~25) replicate spectra at each waiting time to improve the statistics of the CLS decay itself. Finally, in the data that we report here, we sampled the waiting time, T , in 50 fs steps from 0–5 ps, as opposed to the much larger step size used in previous measurements. These improvements have allowed for the observation of features of the FFCF that were unresolvable in previous measurements. One last difference from our previous experiments that we believe also improves the quality of our current measurements is the purity of the enzyme itself. Those experiments used a commercial FDH extracted from yeast (*Candida boidinii*), which we recently found to be a mixture of different isozymes with different masses and different charges.⁹ That mixture likely led to heterogeneous structures and dynamics whose average further masked the true nature of the FFCF. In the current report we use only one of these isoenzymes produced as described elsewhere.⁹

Figure 2 shows CLS decay data for the NAD^+ ternary complex (black circles). Typically, CLS data are fit to a sum of exponential functions:

$$F(T) = \Delta_0^2 e^{-\frac{T}{\tau_0}} + \sum_i \Delta_i^2 e^{-\frac{T}{\tau_i}}. \quad (1)$$

Equation 1, however, is a poor model for the CLS decay data for the NAD^+ ternary complex because of the oscillatory features in the decay. As a result, we use a functional form that includes damped cosines to model these oscillations:

$$F(T) = \Delta_0^2 e^{-\frac{T}{\tau_0}} + \sum_i \Delta_i^2 e^{-\frac{T}{\tau_i}} * \cos(2\pi\omega_i T + \phi_i). \quad (2)$$

Table I summarizes the FFCF parameters that come from fitting the CLS data to (2.) Modeling the data for the NAD^+ complex requires three terms. The first term accounts for

the offset of the data, which arises from azide not sampling all environments within our experimental window. The last two terms involve underdamped oscillations: one with a frequency of 24 cm^{-1} and the other with a frequency of 10 cm^{-1} , with damping times of .64 ps and 1.1 ps, respectively. In order to accurately fit the data, the higher-frequency oscillation has a phase of $\varphi = \pi/2$. No oscillating frequency fluctuations have previously been reported at this timescale for a vibrational chromophore in an enzyme or a protein. It should be noted again that the scatter in the CLS values was such that CLS values from 20–25 replicate spectra at each waiting time are averaged to definitively resolve these oscillations out of the noise.

Oscillating frequency fluctuations have been observed in the FFCF of the OH stretch of HOD in D_2O ,¹⁰ as oscillations of the hydrogen bond modulate the OH stretching frequency. Azide makes several hydrogen bonds with active site residues (Figure 3). Although the frequency fluctuations in FDH occur with a frequency that is an order of magnitude lower than those observed in bulk water, hydrogen-bond fluctuations can occur on longer timescales in some systems such as with interfacial water.¹¹ As a result, we cannot definitively exclude hydrogen-bond fluctuations as a source of the oscillations based on time scale alone. Coherent excitation of low-frequency modes coupled to the high-frequency azide transition could also give rise to oscillations in the FFCF, as is seen in hydrogen-bonded acetic acid dimers.¹² Coherent excitation of low-frequency modes, however, will also cause oscillations in the intensity of the 2D IR spectra as a function of waiting time. Such oscillations are absent, however, in the intensity of the azide signal from FDH complexed with NAD^+ (see SI).

Another possibility is that motions of the nicotinamide ring of the NAD^+ cofactor could be the source of the oscillations we observe in the FFCF. The ring is aromatic so it moves as a single unit, which would explain the low frequency of the observed oscillations. In addition, the positive charge of the nicotinamide means that fluctuations of the ring should perturb the azide stretching frequency. If fluctuations of the nicotinamide ring were responsible for the oscillations, then we would expect that replacing NAD^+ with NADH in the ternary complex should make these oscillations go away as the reduced form of the cofactor has no charge on the ring and is much more flexible suggesting that it will influence azide less and that the motions will be less organized. As seen in Figure 4, the CLS decay for azide bound to the NADH ternary complex exhibits much simpler behavior than for the NAD^+ ternary complex. The oscillations are essentially gone, and the data can be fit with a single exponential decay ($\tau_1=.64\text{ ps}$) and a static component ($\tau_2=\infty$) (Table I). Close inspection of the data shows that the CLS for the NADH complex may exhibit a small amplitude oscillation with a frequency similar to that seen in the NAD^+ complex, but the inclusion of the additional fit parameters required to fit with an additional oscillatory term are not statistically justified as assessed by an F-test ($F=11.3 > F_{\text{crit}}=5.1$).

The observation that the oscillation disappears in the case of the reduced cofactor strongly suggests the oscillations in the FFCF for the complexes with NAD^+ are associated with motions of the charged nicotinamide ring. It is also possible that the oscillations are present in both the NAD^+ and NADH complexes, but they are not observable in the complex with NADH because the nicotinamide ring is uncharged in this complex. Alternatively, it is

possible that the change in the electronic structure of the ring alters the intermolecular interactions enough that the oscillatory motions no longer appear, which is not uncommon when a reactant-like complex is changed to a product-like complex.¹³ In either case, the low frequency of these oscillations is a remarkable result, as motions with such low frequencies should be strongly overdamped as the result of interactions with the many thermally-activated motions of the protein and solvent. The observed oscillations, along with the substantial motional narrowing of the lineshape as compared to chromophores in other protein/enzyme systems,^{14–17} suggest that the enzyme active site protects the azide/NAD⁺ pair from the friction that would normally arise from interactions of azide with the environment. While these data differ from our previous results, they are consistent with the interpretation of a rigid protein cage in which the azide and nicotinamide remain insulated from the thermal noise of the solvent, which has the potential to be functionally relevant because such an environment would facilitate efficient hydrogen tunneling at the tunneling ready state.

In addition to the inherent significance of underdamped oscillations at this timescale, the conclusion that these oscillations involve motions of the NAD⁺ cofactor lends particular significance to these results in the context of the enzyme-catalyzed hydride transfer, as donor-acceptor fluctuations are expected to play an important role in the temperature dependence of kinetic isotope effects on the hydride transfer reaction. Specifically, theoretical models suggest that temperature-dependent kinetic isotope effects on the hydride transfer reflect donor-acceptor distance sampling at femtosecond to picosecond timescales.^{18–20} Our results suggest that we are able to directly probe donor-acceptor fluctuations in a complex that mimics the tunneling ready state for the enzyme-catalyzed hydride transfer. Thus the FDH ternary complex offers an ideal opportunity for studies of the role of donor-acceptor fluctuations in enzyme-catalyzed hydride transfer reactions.

Conclusions

Improvements in 2D IR spectroscopy have allowed a more in depth study of enzyme structural dynamics at the femtosecond to picosecond timescale. Although the details of the results differ, the current study shows that the active site of FDH is well protected from interactions with the thermal noise of the protein and solvent consistent with a fairly rigid active-site cage. Furthermore, our results reveal underdamped oscillatory motions on the picosecond timescale, which are both unexpected and unprecedented in other studies of dynamics in either proteins or solution-phase environments. The evidence in this study suggests that these oscillations are attributable to motions of the charged nicotinamide ring of the NAD⁺ cofactor, though further studies including mutagenesis, isotope labeling of the cofactor and/or the protein, and molecular dynamics simulations would be needed to definitively assign the molecular origins of these motions. This result also motivates further investigation into the role that these motions may play in the reaction catalyzed by FDH, a system that is ideally suited to such studies.

Experimental Methods

The apparatus for 2D IR measurements has been described in detail previously.⁸ Optical parametric amplification in β -barium borate (BBO) followed by difference frequency generation in AgGaS₂ 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 t . 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 t_1 to 4 ps. Following upconversion to the visible, 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 two pump pulses at each time delay to give a purely absorptive 2D IR signal. Fourier transformation with respect to τ_1 gives the ω_{pump} axis. Waiting times range from 0 to 5 ps, in 50 fs steps. To extract the FFCF, the CLS data are fit to a sum of two oscillating exponentials and a decaying exponential:

$$C(T) = A_0 e^{-\frac{T}{\tau_0}} + \sum_i A_i e^{-\frac{T}{\tau_i}} * \cos(2\pi\omega_i T + \phi_i) \quad (3)$$

Where A_i are the absolute amplitudes of the CLS, which are determined by the normalized FFCF and a scaling factor related to the homogeneous dephasing time. The amplitude of each term in the FFCF, A_i , is estimated from the FWHM of the FTIR spectrum using the method provided by Kwak, et al:²¹

$$\Delta_i = \sqrt{\frac{A_i}{\sum_i A_i} \frac{FWHM}{2\sqrt{2\ln 2}}} \quad (4)$$

The dephasing time, T_2 , is also estimated from the FWHM of the FTIR spectrum and the initial value of the CLS:

$$T_2 = \frac{1}{\pi * FWHM * \sqrt{\sum_i A_i}} \quad (5)$$

FDH from *Candida boidinii* is expressed and purified according to previously published procedures.⁹ The FDH-NAD⁺(NADH)-N₃⁻ ternary complex is prepared in 100 mM phosphate buffer at pH 7.5. The final concentrations for FDH, NAD⁺(NADH) and azide are 1.6, 2 and 1.5 mM, respectively. A 5 μ L sample of the ternary complex is placed between CaF₂ windows with a 56 mm spacer in a temperature-controlled cell (Harrick Scientific) and incubated at 5 °C for both 2D IR and FT IR measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. 2D IR spectrum of azide anion bound to FDH and NAD^+ at $T = 25$ ps plotted in absorbance mode as $-\text{OD}$. The purple circles are the centerline points obtained from taking slices in ω_{pump} and finding the maximum value. The solid white line is the linear fit to the centerline. More 2D IR spectra showing the time evolution of the lineshape can be found in the SI

Figure 2. CLS decay data of azide bound to FDH and NAD^+ . The circles are the data and the solid line is the result of the fit in eq. 2.

Figure 3.
Crystal structure of the active site of FDH from *Candida boidinii*, PDB ID: 5DN9.

Figure 4. CLS decay data of azide bound to FDH and NADH. The circles are the data and the solid line is the result of the fit in eq. 1.

Table 1

FFCF parameters for azide bound to ternary complexes of NAD^+ and NADH.

	ω_0 (cm^{-1})	τ_0 (ps)	Γ (cm^{-1})	τ_1 (ps)	ω_1 (cm^{-1})	Γ_1 (cm^{-1})	ω_2 (cm^{-1})	τ_2 (ps)	Γ_2 (cm^{-1})	τ_2 (ps)
NAD^+	1.54 ± 0.04	80 ± 40	0.66 ± 0.05	0.64 ± 0.19	24 ± 1	0.79 ± 0.03	9.9 ± 0.4	1.08 ± 0.13	9.9 ± 0.4	2.4 ± 0.4
NADH	1.46 ± 0.04	∞	1.23 ± 0.03	0.64 ± 0.27	-	-	-	-	-	2.4 ± 0.1