Two-dimensional infrared spectroscopy of azido-nicotinamide adenine dinucleotide in water

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Mid-IR active analogs of enzyme cofactors have the potential to be important spectroscopic reporters of enzyme active site dynamics. Azido-nicotinamide adenine dinucleotide (NAD^+), which has been recently synthesized in our laboratory, is a mid-IR active analog of NAD^+ , a ubiquitous redox cofactor in biology. In this study, we measure the frequency–frequency time correlation function for the antisymmetric stretching vibration of the azido group of azido- NAD^+ in water. Our results are consistent with previous studies of pseudohalides in water. We conclude that azido- NAD^+ is sensitive to local environmental fluctuations, which, in water, are dominated by hydrogen-bond dynamics of the water molecules around the probe. Our results demonstrate the potential of azido- NAD^+ as a vibrational probe and illustrate the potential of substituted NAD^+ -analogs as reporters of local structural dynamics that could be used for studies of protein dynamics in NAD -dependent enzymes. © 2011 American Institute of Physics. [doi:10.1063/1.3623418]

INTRODUCTION

There is growing evidence that femtosecond to picosecond time scale protein dynamics can affect the rate of enzyme-catalyzed hydrogen transfers.^{1–9} With the advent of nonlinear vibrational spectroscopies such as two-dimensional infrared spectroscopy (2D IR), it is possible to explore the structure-dynamics-function relationships of proteins at the time scales most relevant to the catalyzed chemical conversion. The 2D IR spectrum as a function of the waiting time measures the time scales of environmental fluctuations that influence the frequency of a probe vibration. Thus, successful studies of enzyme dynamics using 2D IR depend on the placement of a suitable vibrational probe in the protein in a location where it can sample relevant dynamics. Mid-infrared probes, such as pseudohalide anions, nitriles, and carbon monoxide, are ideal candidates as environmental reporters to monitor conformational fluctuations of proteins on the picosecond to femtosecond time scale because their absorptions fall within the “transparent” window of the protein and water absorption spectra from 1800 cm^{-1} to 2300 cm^{-1} . There have been several reports of protein dynamics measured using these mid-IR active chromophores as chelating agents, substrates, or inhibitors. For example, carbon monoxide has been used to study several heme proteins including myoglobin, hemoglobin, neuroglobin, and horseradish peroxidase.^{10–16} Azide anion has been used to study bovine carbonic anhydrase II,¹⁷ and both wild-type and several mutants of human carbonic anhydrase II,¹⁸ and a transition-state analog complex of formate dehydrogenase.^{1,19} In addition, the nitrile chromophores of the non-nucleoside inhibitor TMC278 have been used to study the dynamics HIV-1 reverse transcriptase.²⁰

In spite of these successes, however, the number of proteins for which femtosecond to picosecond dynamics have been studied is small because only a few proteins have a natural propensity to bind one of these mid-IR active chromophores. Thus the approach of using established probes to study protein dynamics is an inherent limitation on the widespread application of 2D IR spectroscopy to enzyme dynamics because such probes cannot be used with most proteins. One strategy that has been pursued to address this problem is to attach a chromophore to the protein by chemical modification at a desired position within the protein.^{21,22} For example, nitrile or azido substituted amino acids, such as p-cyano-phenylalanine and 5-cyano-tryptophan, which have been incorporated in several proteins and peptides,^{23–28} and azido-alanine^{29,30} and azidohomoalanine,³¹ which have been shown to be sensitive to electrostatic interactions and local peptide conformation. Another approach is to convert cysteine residues in the protein to an IR-active chromophore by chemically modifying the cysteine thiol in the protein to a thiocyanate.^{32–34} Yet another approach involves incorporating deuterated amino acids, such as Met- d_3 and Leu- d_{10} , into the protein site selectively and probing the C-D stretching vibrations of these deuterated amino acids.^{35–40} A major challenge in all of these approaches, however, is that they all involve significant modifications of the protein itself. These modifications can pose two challenges: First, because of the significant protein chemistry involved, it can be difficult to express the modified protein at levels adequate for nonlinear vibrational spectroscopy experiments, which can require tens of milligrams of protein to make a sample for the 2D IR experiment when using a standard infrared transmission cell. Second, the protocol for incorporating the chromophore must be redeveloped for each new protein under study to ensure that the probe is placed at the desired location and that the placement is selective. Although these methods can be

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straightforward, they are not always trivial. Thus, there is a need for spectroscopic probes that can be placed into the active site of a large number of proteins and offer a simple and consistent protocol for incorporating the probe in the active site. One potentially powerful strategy is to introduce labeled ligands, which have been used for probing protein-ligand interaction dynamics and electrostatics previously.^{20,41} A general probe of this type that can be employed without modifying the protein would make a wide range of enzymes accessible for study by 2D IR spectroscopy.

NAD⁺, the related coenzyme 2'-phosphate-NAD⁺ (NADP⁺), and the reduced forms of these cofactors are ubiquitous redox agents for many biological processes, and NAD⁺ is a coenzyme for several supra-families of enzymes comprising many hundreds of proteins. NAD⁺ and NADH bind those enzymes with their nicotinamide rings in the active site in contact with the substrate to be reduced or oxidized in the catalytic reaction. Consequently, nicotinamide-analogs with vibrational probes attached to the nicotinamide ring are likely to be able to report on the dynamics of the active sites of a wide variety of nicotinamide-dependent enzymes. Analogs of NAD⁺ are widely used as mechanistic probes to understand enzymatic pathways,^{42–47} and the idea of using a NAD-analog as a spectroscopic reporter holds great potential as a probe with widespread applications to many important enzymatic systems.

We have recently synthesized a mid-IR active analog of NAD⁺, azido-NAD⁺, that has the potential to be a general probe to investigate active-site dynamics in nicotinamide-dependent enzymes.⁴⁸ Figure 1 compares the structures of NAD⁺ and azido-NAD⁺. In azido-NAD⁺, the amide group on the nicotinamide ring is replaced with an azido group to serve as an infrared reporter that is expected to minimally perturb the interactions with enzymes to which it binds. Of course, like many spectroscopic probes, the binding of this analog might lead to some perturbation relative to the natural cofactor, but the design of this probe is likely to minimize this effect since the azido group is similar in size and hydrophilicity to the original amide. Indeed, studies of this analog with three different enzymes indicated that it is either

an active coenzyme or a competitive inhibitor,⁴⁸ suggesting that it is a rather faithful analog. Nevertheless, as with most spectroscopic probes, caution must be practiced in interpreting the findings about any enzyme under study, keeping in mind that it is an analog and not the natural cofactor. Azido-NAD⁺ has a single infrared transition at 2140 cm⁻¹, which has potential as a reporter of active site dynamics. In aqueous solution, the line shape of this transition broadens to 33 cm⁻¹ while the typical line width for the enzyme-bound analog is 26–28 cm⁻¹. In addition, the azido antisymmetric stretching vibration is known for having a large transition dipole moment. It seems likely that this chromophore can be a viable probe to study enzyme dynamics, but it is necessary to determine its population lifetime and to assess its sensitivity to solvent dynamics to confirm its utility. In this work, we report 2D IR spectroscopy of this probe in phosphate buffer to characterize the aqueous solvation dynamics experienced by the azido group in azido-NAD⁺. Our results suggest that hydrogen bonding fluctuations of the hydrogen-bonded waters in the first solvation shell likely make the dominant contribution to the observed dynamics. Furthermore, our results demonstrate the feasibility of using azido-NAD⁺ as a probe of environmental fluctuations and suggest that it should be a good probe of protein dynamics in NAD(P)-dependent enzymes.

EXPERIMENTAL METHODS

For time-resolved infrared spectroscopy experiments, an amplified Ti:Sapphire laser system (Spectra Physic, Spitfire) generates 800 nm pulses of 80 fs duration with ~4 mJ of energy per pulse at a 1 kHz repetition rate. A portion of this output, about 800 μ J per pulse, pumps an optical parametric amplifier (OPA) based on a β -barium borate (BBO) crystal ($\theta = 27^\circ$, type II). The OPA generates two tunable near-infrared pulses, signal and idler, with a combined pulse energy of 270 μ J. The signal and idler are tuned such that the difference in the energies of the photons from each beam is equivalent to the energy of the mid-infrared light needed to excite the azido-NAD⁺ sample, 2140 cm⁻¹. These are then mixed in an AgGaS₂ difference frequency generation (DFG) crystal ($\theta = 50^\circ$, type II) that produces nearly transform limited pulses with a pulse duration of ~100 fs and a pulse energy of 6 μ J.

For infrared pump-probe experiments, a wedged CaF₂ substrate separates a small portion of the infrared light for use as a probe beam. The remaining infrared light is used as a pump beam. An infrared pulse shaper based on a germanium acousto-optic modulator built according to the design of Shim *et al.*^{49,50} chops the pump beam on alternating laser shots. The throughput of the pump pulse is such that, at the sample, the pump pulse energy is 1 μ J. A half-wave plate and polarizer in the pump beam set the polarization of the pump at the magic angle relative to the probe beam to remove the reorientation contribution to the signal. A computer-controlled translation stage varies the time delay between pump and probe pulses. The pump and probe beams are aligned parallel to one another separated by one half inch. A 90° off-axis parabolic mirror with an effective focal length of 100 mm focuses the beams into the sample where they have a spot size of ~80 μ m. After a second parabolic mirror recollimates the beams, an iris

FIG. 1. Structures for nicotinamide adenine dinucleotide (NAD⁺), left, and azido-nicotinamide adenine dinucleotide (azido-NAD⁺), right.

isolates the probe beam. A zero-dispersion stretcher with a slit at the Fourier plane frequency narrows a portion of the original 800 nm light to a transform-limited bandwidth of ~ 1 cm^{-1} . The narrowed 800 nm beam upconverts the infrared probe beam in a MgO-doped LiNbO₃ crystal ($\theta = 46.5^\circ$, type I) into the visible. An imaging spectrometer (300 mm focal length, 1200 grooves/mm grating) disperses the upconverted probe beam and a CMOS camera with 1024 pixels measures the spectrum of each individual laser shot. Using adjacent probe spectra, we calculate the change in optical density caused by the pump beam.

For the 2D IR experiments, three mid-IR excitation pulses of 120 fs duration focus in a sample cell. Computer-controlled translation stages vary the time delays between the pulses. The time between the first and second pulses is the evolution time (τ) and that between the second and third pulses is the waiting time (T). The interaction of these three pulses with the sample generates a response in a unique direction determined by the phase matching condition, $-\mathbf{k}_1 + \mathbf{k}_2 + \mathbf{k}_3$. The signal overlaps spatially and temporally with a local oscillator beam for heterodyned detection of the amplitude and phase of the signal electric field. A monochromator spectrally disperses the signal and local oscillator, and an MCT detector measures the intensity at each wavelength. Interferograms in the evolution time are collected at each signal wavelength for fixed values of the waiting time. The interferograms are Fourier transformed to give frequency spectra along the horizontal axis (ω_1) at each monochromator position, which provides the vertical axis (ω_3) of the final 2D-IR spectrum.

The detailed synthesis of azido-NAD⁺ is described in Ref. 48. In short, we synthesize 3-azidopyridine following the method described by Sawanishi *et al.*⁵¹ We then synthesize azido-NAD⁺ by exchanging the nicotinamide ring of the natural coenzyme for 3-azidopyridine using pig brain NADase. We extract the resulting solution with chloroform to remove any remaining azidopyridine, and then separate the remaining components in the aqueous phase by chromatography using C-18 silica gel with a methanol-water mixture as the solvent. The solid azido-NAD⁺ is finally isolated by rotary evaporation and lyophilization of the fractions that are richest in azido-NAD⁺ as identified by the characteristic absorption at 303 nm. The sample cell is composed of two 3 mm thick CaF₂ windows separated by an optical path length of 25 μm and containing a solution of 50 mM azido-NAD⁺ in H₂O.

RESULTS AND DISCUSSION

Figure 2 shows spectral slices from the dispersed pump-probe measurement of azido-NAD⁺ in water at time delays of 500 fs (red), 1 ps (blue), and 2 ps (black). The positive-going feature is the excited-state absorption and the negative going feature, which we will refer to as the ground-state bleach, represent contributions from both the ground-state bleach and stimulated emission from the excited state. As can clearly be seen in these slices, the excited-state absorption decays slightly faster than the ground-state bleaching contribution. This result is expected since the initial population relaxation subsequently populates states of the molecule that are anhar-

FIG. 2. Spectral slices of the dispersed pump-probe decay for delay times $T = 500$ fs (red), 1 ps (blue), and 2 ps (black).

monically coupled to the azido antisymmetric stretch. Thus, the ground state does not recover on the same time scale that the excited state relaxes. Further evidence for this anharmonic coupling is given by the time dependence of the peak position of the ground-state bleaching feature, which shifts slightly to higher frequency as a function of the pump-probe time delay as a result of the small anharmonicity of the intermediate states that are populated by the initial relaxation of the excited state. Figure 3 shows the transient population decay for the pixel corresponding to the maximum of the excited-state-absorption signal along with the fit to the population relaxation. This transient is fit well by a single exponential with a time constant of 1.0 ± 0.1 ps. This time scale is in reasonable agreement with what has been seen in previous studies of azido stretching vibrations.

Figure 4 shows 2D IR spectra of the azido stretching transition of azido-NAD⁺ at three different waiting times: $T = 0$ fs, 500 fs, and 1100 fs. The red lobe represents increased transmission as a result of ground-state bleaching and stimulated emission, whereas the blue lobes represent decreased transmission corresponding to the excited state absorption. Because azido-NAD⁺ is a relatively weak chromophore, $\epsilon = 250 \text{ M}^{-1} \text{ cm}^{-1}$ the signal in these spectra is

FIG. 3. Decay of the excited-state absorption contribution to the dispersed pump-probe spectrum as a function of the pump-probe delay. The transient is fit to a single exponential decay with a time constant of 1.0 ps.

weak compared to that for the azide anion at similar sample concentrations. In addition, the population lifetime is rather short. Thus the signal-to-noise at waiting times a picosecond or longer can be poor. This effect is seen clearly in the data for $T = 1100$ fs where the amplitude of the noise is significant relative to that for the signal, as seen in the features above and below the two lobes corresponding to the signal. At early waiting times, the lobes are elongated along the diagonal indicating a strong correlation between the frequency ω_1 and the frequency ω_3 measured after the waiting time. At later waiting times, however, the lobes rotate toward horizontal indicating the loss of correlation between the frequency ω_1 and the frequency ω_3 . This loss of correlation and the corresponding change in peak shape reflect spectral diffusion resulting from the equilibrium environmental fluctuations. The two-point frequency–frequency time correlation function (FFCF), which is given by $\langle \delta\omega(t)\delta\omega(0) \rangle$, quantifies the underlying dynamics. To extract the FFCF from our 2D IR data, we use the centerline slope (CLS) method first introduced by the Fayer group.^{52,53} In this method, we take slices through the 2D spectrum at each value of ω_1 and locate the position of the maximum in ω_3 . The resulting sequence of points is the centerline, and the slope of a linear fit to this line is proportional to the FFCF for this value of T . By measuring the CLS as a function of T , we can quantify the time scales for the structural fluctuations in the environment of the chromophore. Following the method described by Kwak *et al.*, we then use the relative amplitudes of the exponential decay and the time scales of that decay to fit the infrared absorption line shape to determine the FFCF.⁵³ In Fig. 4, the open blue circles show the center line for each spectrum along with the fit (red line).

Figure 5 shows the CLS as a function of T . We fit the decay of the CLS to a single exponential, which corresponds to a simple Kubo line shape model. Although we have collected data for waiting times between 0 and 150 fs, we only fit the CLS decay for times after the initial 150 fs because there is significant distortion of the 2D IR line shape and thus of the magnitude of the CLS as a result of responses that arise from the solvent and the sample windows during the times when our pulses overlap. Fitting the decay of the CLS to a single exponential yields a time constant of 655 ± 100 fs. We then fit the infrared absorption line shape to determine the FFCF according to the procedure outlined by Kwak *et al.*⁵³ To do the fitting, we assume a Kubo line shape model for the FFCF, $C(t) = \Delta^2 e^{-t/\tau}$, and we include a homogeneous contribution to the line shape, Γ , associated with the fast structural fluctuations in the motionally narrowed limit. We also include a contribution from the excited state population relaxation, which we determine from the pump-probe decay. The parameters that result from the fitting are $\Gamma = 12$ cm⁻¹, $\Delta = 2.0$ ps⁻¹, and $\tau = 655$ fs. The inset of Fig. 5 compares the calculated and the experimental absorption spectra of azido-NAD⁺. The calculated spectrum is in excellent agreement with the measured spectrum.

The FFCF for azido-NAD⁺ reflects similar dynamics to those seen previously for aqueous solutions. The time scale of the exponential decay of the FFCF, $\tau = 655$ fs, is very similar to the FFCF decay seen for cyanide anion in water where the correlation function decays with a 464 fs time constant. For

FIG. 4. 2D-IR spectra of azido-NAD⁺ for population times $T = 0$ fs, 500 fs, and 1.1 ps. The open circles mark the center lines and the solid red lines are the linear fits to the centerlines that we use to determine the CLS.

cyanide, this time scale was suggested to involve the weakening and strengthening of hydrogen bonds between water and the anion. This interpretation is consistent with the interpretation of the FFCF decay for azide in D₂O as well.^{54,55} By analogy with these previous studies, we suggest that the dynamics represented by the 655 fs decay in the FFCF of azido-NAD⁺ likely correspond to local fluctuations of the hydrogen bonds between water and the azido group.

The π -stacking interaction of the two bases of azido-NAD⁺ is another potential contribution to the azido vibrational dynamics. In aqueous solution, on average, the adenine

FIG. 5. CLS vs waiting time (open circles) along with the exponential fit to the CLS decay. The inset compares the measured (red) and calculated (black) antisymmetric stretch absorption spectrum of the azido-NAD⁺ chromophore. The structure of Azido-NAD⁺ is also shown (ADPR: adenine pyrophosphate).

and pyridine rings lie parallel to one another to take advantage of favorable π -stacking interactions between the rings. Although this motif is the favored conformation for the natural coenzyme in water, it exists in equilibrium with a minor unfolded conformer. We see no apparent contribution to the FFCF that would correspond to the slow exchange between the open and closed conformers of azido-NAD⁺. As a result, we conclude that local hydrogen bond dynamics make the major contribution to the FFCF decay dynamics that we observe. This result provides the general framework within which the dynamics of this compound should be interpreted when it is enzyme bound.

Azides are sensitive probes of environmental dynamics and organic azides hold great potential as a way to locate a spectroscopic probe in a complex chemical environment where it can report on the structure and dynamics of the system of interest. Azido-NAD⁺ provides a potential tool to investigate the active sites of numerous NAD-dependent enzymes. Our results indicate that this approach holds promise. Unfortunately, azido-NAD⁺ has one major drawback that may ultimately limit its applicability for studies of enzyme dynamics, the molar absorptivity. Specifically, the molar absorptivity for the azido stretching vibration of azido-NAD⁺ is only $\epsilon = 250 \text{ M}^{-1} \text{ cm}^{-1}$, which is smaller than that for the azide anion by almost a factor of 10. Although ϵ is proportional to μ^2 , the 2D IR signal scales as μ^4 , so the decreased transition moment poses a substantial experimental challenge, and efforts to measure the dynamics of this probe bound to an enzyme are ongoing. The origin of this decrease in transition moment is likely to involve the interaction of the azido electronic structure with the aromatic ring. A similar decrease in the transition moment is observed for azidobenzene.⁴¹ The

same study showed, however, that the insertion of a methylene between the azido group and the ring resulted in a significant increase in the transition moment of the azido stretch suggesting that such a modification of azido-NAD⁺ might also recover the strong transition moment that would be needed to make studies of the modified NAD⁺ analog bound to enzymes easier. Another potential shortcoming of azido-NAD⁺ as a probe is that it exhibits a short population lifetime that would only be adequate to sample the dynamics of enzymes up to a few picoseconds. Because the population relaxation of both the azide anion and organic azides are seen to occur on similar time scales, it is likely that the lifetime that we observe for azido-NAD⁺ is characteristic of the azido stretching vibration in other contexts as well. To improve the time scales over which we can probe dynamics using a NAD analog of this sort, we should explore other pseudohalides that can be attached at this site such as $-\text{CH}_2\text{N}_3$, $-\text{CN}$, $-\text{OCN}$, $-\text{SCN}$, or $-\text{SeCN}$, some of which may exhibit longer population lifetimes and provide the possibility to explore protein dynamics in NAD-dependent enzymes over much longer time scales.

In spite of these potential shortcomings, however, the azido-NAD⁺ has distinct advantages as a mid-IR probe suggesting that this approach holds much promise for future investigations of enzyme dynamics. The mid-IR transition of this analog is at 2140 cm^{-1} , which is located in the “transparent” window of the aqueous protein absorption spectrum that can be accessed by nonlinear infrared spectroscopies. It forms stable complexes with several enzymes that catalyze a variety of redox reactions in biological systems, and kinetic studies show that it not only binds but also that, in some cases, it retains activity as a substrate for the enzyme.⁴⁸ Together with our current spectroscopic studies, it seems likely that modifying NAD⁺ by replacing the amide group on the nicotinamide ring with a spectroscopic reporter such as azide is a promising approach for developing spectroscopic probes of enzyme dynamics with widespread applicability for numerous NAD-dependent dehydrogenases.

CONCLUSION

Using cofactors as mid-IR probes has great potential, as they open exciting new areas of physical study of biological systems. The results presented here suggest that azido-NAD⁺ will be sensitive to local environmental fluctuations particularly those of its hydrogen bonding partners. In this study of azido-NAD⁺ in water, we observe dynamics that are consistent with the time scale for fluctuations of the hydrogen bond that water makes with the azido group. In fact, the FFCF is very similar to that for other small chromophores previously studied to understand the solvation dynamics of water. Thus, our work shows that derivatized analogs of NAD⁺ should be powerful probes of the protein dynamics of NAD-dependent enzymes.

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- ¹J. Bandaria, C. M. Cheatum, and A. Kohen, *J. Am. Chem. Soc.* **131**(29), 10151 (2009).
- ²L. Wang, N. M. Goodey, S. J. Benkovic, and A. Kohen, *Philos. Trans. R. Soc. London, Ser. B* **361**(1472), 1307 (2006).
- ³O. A. Oyeemi, K. M. Sours, T. Lee, K. A. Resing, N. G. Ahn, and J. P. Klinman, *Proc. Natl. Acad. Sci. U.S.A.* **107**(22), 10074 (2010).
- ⁴J. P. Klinman, *Chem. Phys. Lett.* **471**(4–6), 179 (2009).
- ⁵S. Hay, C. R. Pudney, T. A. McGrory, J. Y. Pang, M. J. Sutcliffe, and N. S. Scrutton, *Angew. Chem., Int. Ed.* **48**(8), 1452 (2009).
- ⁶J. Y. Pang, S. Hay, N. S. Scrutton, and M. J. Sutcliffe, *J. Am. Chem. Soc.* **130**(22), 7092 (2008).
- ⁷A. Dybala-Defratyka, P. Paneth, R. Banerjee, and D. G. Truhlar, *Proc. Natl. Acad. Sci. U.S.A.* **104**(26), 10774 (2007).
- ⁸S. D. Schwartz and V. L. Schramm, *Nat. Chem. Biol.* **5**(8), 552 (2009).
- ⁹D. Antoniou, J. Basner, S. Nunez, and S. D. Schwartz, *Adv. Phys. Org. Chem.* **41**, 315 (2006).
- ¹⁰H. Ishikawa, S. Kim, K. Kwak, K. Wakasugi, and M. D. Fayer, *Proc. Natl. Acad. Sci. U.S.A.* **104**(49), 19309 (2007).
- ¹¹H. Ishikawa, I. J. Finkelstein, S. Kim, K. Kwak, J. K. Chung, K. Wakasugi, A. M. Massari, and M. D. Fayer, *Proc. Natl. Acad. Sci. U.S.A.* **104**(41), 16116 (2007).
- ¹²I. J. Finkelstein, H. Ishikawa, S. Kim, A. M. Massari, and M. D. Fayer, *Proc. Natl. Acad. Sci. U.S.A.* **104**(8), 2637 (2007).
- ¹³A. M. Massari, I. J. Finkelstein, and M. D. Fayer, *J. Am. Chem. Soc.* **128**(12), 3990 (2006).
- ¹⁴I. J. Finkelstein, A. Goj, B. L. McClain, A. M. Massari, K. A. Merchant, R. F. Loring, and M. D. Fayer, *J. Phys. Chem. B* **109**(35), 16959 (2005).
- ¹⁵B. L. McClain, I. J. Finkelstein, and M. D. Fayer, *Chem. Phys. Lett.* **392**(4–6), 324 (2004).
- ¹⁶B. L. McClain, I. J. Finkelstein, and M. D. Fayer, *J. Am. Chem. Soc.* **126**(48), 15702 (2004).
- ¹⁷M. H. Lim, P. Hamm, and R. M. Hochstrasser, *Proc. Natl. Acad. Sci. U.S.A.* **95**(26), 15315 (1998).
- ¹⁸S. E. Hill, J. Bandaria, M. Fox, E. Vanderaugh, A. Kohen, and C. M. Cheatum, *J. Phys. Chem. B* **113**(33), 11505 (2009).
- ¹⁹J. N. Bandaria, S. Dutta, S. E. Hill, A. Kohen, and C. M. Cheatum, *J. Am. Chem. Soc.* **130**(1), 22 (2008).
- ²⁰C. Fang, J. D. Bauman, K. Das, A. Remorino, E. Arnold, and R. M. Hochstrasser, *Proc. Natl. Acad. Sci. U.S.A.* **105**(5), 1472 (2008).
- ²¹S. S. Andrews and S. G. Boxer, *J. Phys. Chem. A* **104**(51), 11853 (2000).
- ²²J. K. Chin, R. Jimenez, and F. E. Romesberg, *J. Am. Chem. Soc.* **123**, 2426 (2001).
- ²³M. J. Tucker, Z. Getahun, V. Nanda, W. F. DeGrado, and F. Gai, *J. Am. Chem. Soc.* **126**(16), 5078 (2004).
- ²⁴Z. Getahun, C. Y. Huang, T. Wang, B. De Leon, W. F. DeGrado, and F. Gai, *J. Am. Chem. Soc.* **125**(2), 405 (2003).
- ²⁵M. M. Waegle, M. J. Tucker, and F. Gai, *Chem. Phys. Lett.* **478**(4–6), 249 (2009).
- ²⁶K. C. Schultz, L. Supekova, Y. Ryu, J. Xie, R. Perera, and P. G. Schultz, *J. Am. Chem. Soc.* **128**(43), 13984 (2006).
- ²⁷A. T. Fafarman and S. G. Boxer, *J. Phys. Chem. B* **114**(42), 13536 (2010).
- ²⁸A. T. Fafarman, P. A. Sigala, D. Herschlag, and S. G. Boxer, *J. Am. Chem. Soc.* **132**(37), 12811 (2010).
- ²⁹K. I. Oh, J. H. Lee, C. Joo, H. Han, and M. Cho, *J. Phys. Chem. B* **112**(33), 10352 (2008).
- ³⁰J. H. Choi, K. I. Oh, and M. H. Cho, *J. Chem. Phys.* **129**(17), 174512 (2008).
- ³¹H. Taskent-Sezgin, J. Chung, P. S. Banerjee, S. Nagarajan, R. B. Dyer, I. Carrico, and D. P. Raleigh, *Angew. Chem. Int. Ed.* **49**(41), 7473 (2010).
- ³²A. T. Fafarman, L. J. Webb, J. I. Chuang, and S. G. Boxer, *J. Am. Chem. Soc.* **128**(41), 13356 (2006).
- ³³H. A. McMahon, K. N. Alfieri, C. A. A. Clark, and C. H. Londergan, *J. Phys. Chem. Lett.* **1**(5), 850 (2010).
- ³⁴L. Edelstein, M. A. Stetz, H. A. McMahon, and C. H. Londergan, *J. Phys. Chem. B* **114**(14), 4931 (2010).
- ³⁵J. Zimmermann, K. Gundogdu, M. E. Creemeens, J. N. Bandaria, G. T. Hwang, M. C. Thielges, C. M. Cheatum, and F. E. Romesberg, *J. Phys. Chem. B* **113**(23), 7991 (2009).
- ³⁶S. R. G. Naraharisetty, V. M. Kasyanenko, J. Zimmermann, M. C. Thielges, F. E. Romesberg, and I. V. Rubtsov, *J. Phys. Chem. B* **113**(14), 4940 (2009).
- ³⁷M. E. Creemeens, J. Zimmermann, W. Yu, P. E. Dawson, and F. E. Romesberg, *J. Am. Chem. Soc.* **131**(16), 5726 (2009).
- ³⁸M. C. Thielges, D. A. Case, and F. E. Romesberg, *J. Am. Chem. Soc.* **130**(20), 6597 (2008).
- ³⁹C. S. Kinnaman, M. E. Creemeens, F. E. Romesberg, and S. A. Corcelli, *J. Am. Chem. Soc.* **128**(41), 13334 (2006).
- ⁴⁰M. E. Creemeens, H. Fujisaki, Y. Zhang, J. Zimmermann, L. B. Sagle, S. Matsuda, P. E. Dawson, J. E. Straub, and F. E. Romesberg, *J. Am. Chem. Soc.* **128**(18), 6028 (2006).
- ⁴¹I. T. Suydam and S. G. Boxer, *Biochemistry* **42**(41), 12050 (2003).
- ⁴²B. M. Anderson and C. D. Anderson, *Anal. Biochem.* **140**(1), 250 (1984).
- ⁴³J. R. Barrio, J. A. Secrist, and N. J. Leonard, *Proc. Natl. Acad. Sci. U.S.A.* **69**(8), 2039 (1972).
- ⁴⁴A. F. Hartog and J. A. Berden, *FEBS Lett.* **261**(1), 161 (1990).
- ⁴⁵V. N. Hingorani, L. F. H. Chang, and Y. K. Ho, *Biochemistry* **28**(18), 7424 (1989).
- ⁴⁶H. Kim and B. E. Haley, *J. Biol. Chem.* **265**(7), 3636 (1990).
- ⁴⁷F. Schuber, P. Travo, and M. Pascal, *Bioorg. Chem.* **8**(1), 83 (1979).
- ⁴⁸S. Dutta, R. J. Cook, J. C. D. Houtman, A. Kohen, and C. M. Cheatum, *Anal. Biochem.* **407**(2), 241 (2010).
- ⁴⁹S. H. Shim, D. B. Strasfeld, Y. L. Ling, and M. T. Zanni, *Proc. Natl. Acad. Sci. U.S.A.* **104**(36), 14197 (2007).
- ⁵⁰S. H. Shim, D. B. Strasfeld, and M. T. Zanni, *Opt. Express* **14**(26), 13120 (2006).
- ⁵¹H. Sawanishi, K. Tajima, and T. Tsuchiya, *Chem. Pharm. Bull.* **35**, 4101 (1987).
- ⁵²K. Kwak, S. Park, I. J. Finkelstein, and M. D. Fayer, *J. Chem. Phys.* **127**(12), 124503 (2007).
- ⁵³K. Kwak, D. E. Rosenfeld, and M. D. Fayer, *J. Chem. Phys.* **128**(20), 204505 (2008).
- ⁵⁴P. Hamm, M. Lim, and R. M. Hochstrasser, *Phys. Rev. Lett.* **81**(24), 5326 (1998).
- ⁵⁵S. Z. Li, J. R. Schmidt, A. Piryatinski, C. P. Lawrence, and J. L. Skinner, *J. Phys. Chem. B* **110**(38), 18933 (2006).