Published in final edited form as:

Chem Phys Lett. 2009 January 1; 473(4-6): 330–335.

Three-pulse photon echo peak shift spectroscopy as a probe of flexibility and conformational heterogeneity in protein folding

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Abstract

We investigate the equilibrium unfolding of Zn-cytochrome c in guanidine hydrochloride by three-pulse photon echo peak shift (3PEPS) spectroscopy. Unexpectedly, the measurements reveal that inhomogeneous broadening of the sample at the midpoint of the denaturation is larger than that of either native or unfolded states. To interpret this finding, we present simulations of the peak shift for both two-state and three-state unfolding models. Both the denaturant concentration dependence of the asymptotic peak shift (APS) and the wavelength dependence of the APS at the midpoint of the denaturation are different for the two models. Our data are consistent with two-state unfolding.

1. Introduction

The current understanding of protein folding is dominated by the concept of a ‘folding landscape’ for each system [1]. One challenge is to obtain knowledge of the topology for this landscape under various denaturant and temperature conditions. Experimental studies glean information on the landscape following two main categories of approaches. Kinetics measurements employing fluorescence [2], Raman scattering [3], circular dichroism [4], or X-ray scattering [5] provide information on the mechanism, such as activation energies, and the presence of traps or intermediates. On the other hand, structural [6] and thermodynamic [7] studies of equilibrium species provide information on the structures of possible kinetic intermediates, and on stabilities of conformers. It is characteristic of folding that a conformationally diverse ensemble of structures progressively loses this diversity, as it assumes the much smaller set of conformations representing the folded state [8]. This structural diversity leads to kinetic complexity, as it has been suggested that sub-populations fold at different rates [9]. Unfortunately, most techniques are either not capable of quantifying disorder in folding, because they provide average values of a spectroscopic or structural parameter, or they are not applicable to highly disordered states (*e.g.* NMR). Here we take advantage of femtosecond nonlinear spectroscopy, which is applicable to disordered systems due to its ability to characterize the dynamics in a wide range of timescales. Specifically we use three-pulse photon echo peak shift (3PEPS) measurements, to provide insight into the folding of Zn (II)-substituted cytochrome c (Zn-cyt c).

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3PEPS is an electronic four-wave mixing technique that has been used to characterize dynamics in solvents and solutes [10–14], glasses [13,15] and proteins [16–18]. 3PEPS is sensitive to the spectrum of fs–ns motions of the system, which can be regarded as a measure of flexibility, while also having the ability to quantify the inhomogeneous broadening of the electronic transition. This broadening is a measure of conformational diversity.

3PEPS measurements typically reveal several timescales of collective coordinates corresponding to protein motions coupled to the electronic transition. Comparisons of timescales observed for various proteins binding the same cofactors (*e.g.* antibodies) have revealed differences in their flexibilities. 3PEPS has a number of advantages compared to transient absorption or emission measurements: (1) the peak shift decay is typically insensitive to the decay of excited state population, so the complex fluorescence decay kinetics usually observed in proteins are not convoluted with timescales of protein motions; (2) fluorescent cofactors are not necessary; (3) 3PEPS can be used to quantify inhomogeneous broadening, which characterizes the static disorder of the system. From a model based on impulsive excitation of a two-level system coupled to a classical bath of harmonic oscillators [10], it is found that the initial peak shift, $\tau^*(T=0)$, is related to the homogeneous linewidth, Γ , *i.e.* $\tau^*(T=0) \approx (\Gamma\pi)^{-1/2}$, while at long delay times, the asymptotic peak shift (APS), $\tau^*(T \rightarrow \infty)$, is related to the static inhomogeneous linewidth, Δ_{in} , *i.e.* $\tau^*(T \rightarrow \infty) \propto \Delta_{in}^2 (\Gamma + \Delta_{in}^2)^{-1}$, where T is the population time. The peak shift decay timescales reflect those in the time-correlation function of the electronic energy gap. A more complete analysis using realistic excitation pulses and including the high frequency vibrations of the chromophore is available [19,20].

We have investigated the folding of Zn-cyt c with 3PEPS. Zn-cyt c has been studied extensively as it is structurally homologous to the native protein (Fe-cyt c), yet has different porphyrin ligation states. In particular, for denatured horse-heart cyt c at neutral pH, a non-native axial ligand (His26 or His33) replaces the native Met80 [21]. The rate-limiting step of folding is the correction of this mis-ligation [22]. Replacement of Fe by Zn eliminates the possibility of forming these misligated species, because Zn is normally five-coordinate [23]. Furthermore, the metal substitution permits spectroscopic probing of the conformation as the long-lived Q-band excited state of Zn(II) porphyrins opens a several ns window for observing the protein dynamics, compared to Fe(II) or Fe(III) heme, which internally converts back to the ground state within a few picoseconds.

2. Experimental methods

Zn-cyt c was prepared from horse-heart cytochrome c, according to published methods [24]. Denatured and midpoint unfolded Zn-cyt c samples were prepared by adding guanidine hydrochloride (GuHCl) to pH 7, 100 mM phosphate buffer solution to obtain concentrations of 4.5 and 2.5 M, respectively. Denaturant-dependent UV–vis spectra were measured with a double-beam spectrophotometer. The concentration of GuHCl of each sample was precisely determined using a refractometer.

Experiments were performed with the second harmonic of a cavity-dumped, mode-locked Ti:sapphire oscillator. A laser repetition rate of 40 kHz was used to avoid artifacts associated with Zn-porphyrin triplet states. Pulses were tunable from 400 to 425 nm with a bandwidth of 6.5–7 nm, and dispersive broadening in the optics was compensated with a prism pair to provide pulse durations of 43–45 fs at the sample. The time bandwidth product is 0.47–0.5, which is slightly higher than the value for a transform limited Gaussian pulse of 0.44. 3PEPS measurements were performed with an arrangement described previously [25]. The excitation wavelength for each measurement was tuned several nanometers to the red side of the Soret band for each sample. Sample concentrations of 150 μM (corresponding to an optical density of 0.7 at the peak of the absorption band) in a 0.25 mm path-length spinning cell were used.

Christensson et al. have recently shown that high optical density samples and/or long path length cells may result in a signal distortion that artificially increases the asymptotic peak shift [26]. Based on their findings we expect that our conditions may result in an observable increase in the asymptotic peak shift of less than 1 fs, relative to measurements on lower density samples.

3. Experimental results

The 3PEPS data for Zn-cyt c for three denaturant conditions folded, midpoint, and unfolded (0, 2.5, and 4.5 M GuHCl), are shown in Fig. 1. The data were fit by nonlinear least squares to a functional form with multiple exponential decay components, reported in Table 1(a). All traces have a damped sinusoidal component with a frequency of 152 cm^{-1} and damping time constant of 50–58 fs. This oscillation results from a vibrational mode of the Zn-heme [27]. According to Champion's result on Fe-heme [28], this mode is sensitive to the local heme environment and is therefore expected to change upon unfolding. However, due to the short damping timescale and the signal to noise, we do not have the resolution to make a conclusion. The timescale of the fastest (30–50 fs) decay component is coupled to the fit of the oscillatory mode, but in similar protein/cofactor systems, this timescale results from high frequency chromophore and protein motions [17]. Experimentally, the initial peak shift is affected by pulse duration, detuning of the laser, chirp of pulse [20] and small errors in determination of $T = 0$. The 10% difference between the initial peak shifts in the three traces is dominated by these factors. The most interesting changes are observed at longer timescales. In particular, the increase in the intermediate time constant of the decay upon unfolding corresponds to an increase in flexibility (lower frequency motions) of the unfolded conformer. The substantial increase in the asymptotic peak shift indicates a larger static inhomogeneous broadening, which is consistent with the larger conformational diversity expected of an unfolded protein relative to the native state. The midpoint shows a larger asymptotic peak shift than native or unfolded states, indicating a larger inhomogeneous broadening relative to the other two conformations.

In the next section, we will show that two and three-state unfolding models, since they imply different conformational distributions, lead to different behaviors of the inhomogeneous broadening observed by 3PEPS. We now consider the UV-vis absorption spectra of the heme cofactor, which is a sensitive probe of protein conformation [29], and can be analyzed to obtain relative conformer populations with varying denaturant concentration. The Soret-band spectra (inset of Fig. 1) blue-shifts from 422 to 416 nm (shift of 342 cm^{-1}) and broadens from 907 to 1050 cm^{-1} full width half maximum (FWHM) upon unfolding. The midpoint spectrum is slightly broader (1162 cm^{-1} FWHM) than that of the unfolded form. Fig. 2 shows the entire spectral change of Zn-cyt c with varying GuHCl concentration. Following the method of Latypov et al. [29], we fit the measured spectra of Fig. 2 to both two- and three-state unfolding models to interpret the conformer population as a function of denaturant concentration. The two- and three-state models are described by the following equilibrium relations:

Two-state:

Three-state:

In the two-state model, the native conformer (N) directly unfolds to the unfolded conformer (U) whereas in the three-state model, an intermediate conformer (I) is present. All the transitions, (N-to-U, N-to-I, I-to-U) are described by a midpoint concentration $C_{m(1,2)}$ and slope

$m_{(1,2)}$ that describes a linear dependence of the Gibbs free energy on denaturant concentration. Fig. 3 shows the absorbance as a function of GuHCl concentration for a series of wavelengths. These series of data were simultaneously fit to solve for C_m and m values using the global fitting routine in IGOR Pro. For the fits to both two- and three-state models, data sets of 51 traces (385–435 nm with 1 nm increments) were used, and selected wavelengths are displayed in Fig. 3a and b. Fits were performed for both the two- and three-state models for comparison (solid lines in Fig. 3a and b). In order to obtain reasonable fits, the spectrum of the unfolded conformer was allowed to vary with denaturant concentration (to reflect solvent interactions with the heme in the unfolded state). On the other hand, for a reasonable fit for the three-state model, the spectrum of the native conformer was held fixed during the fitting routine. As shown in Fig. 3a, the two-state model can reasonably interpret the curves, particularly when including the solvent dependent shift of the unfolded spectrum. While the three-state model (Fig. 3b) fits the data slightly better (chi square values are: 0.00212 for three-state and 0.0193 for two-state), the UV-vis spectra do not provide convincing evidence for one model or the other. The best fit parameters are: $C_m = 2.54$ M, $m/RT = 1.43$ M⁻¹ for two-state model and $C_{m1} = 0.91$ M, $m_1/RT = 2.71$ M⁻¹; $C_{m2} = 2.48$ M, $m_2/RT = 4.80$ M⁻¹ for three-state model, with which we can calculate the population of each conformer for a given GuHCl concentration, as shown in Fig. 3c and d. The midpoint of the titration for two-state model is approximately 2.5 M GuHCl; thus, we use this concentration for our 3PEPS investigation.

4. Simulations

The nonlinear response function formalism [30] was utilized to interpret the experimental data, with the specific aim of determining the extent to which 3PEPS can distinguish between the different unfolding models. Simulations of photon echo experiments by this method, also employing the high temperature approximation, have been described in many publications [11,19]. Here, we use this approach with modifications to account for the non-Gaussian inhomogeneous broadening due to the distribution of conformers. We simulated the APS as a function of GuHCl concentration and pump laser wavelength for both two- and three-state models.

In our simulation, the line broadening function is expressed as:

$$g(t) = i\lambda \int_0^t dt_1 M(t_1) + \Delta^2 \int_0^t dt_1 \int_0^{t_1} dt_2 M(t_2) + (\Delta_{in} t)^2 / 2,$$

where $M(t)$ is the time-correlation function of the electronic transition frequency. In our previous work, we gave the $M(t)$ for the native Zn-cyt c [25], as a sum of Kubo oscillators and damped vibration oscillators:

$$M(t) = \Delta_1^2 \exp\left(-\frac{t}{\tau_1}\right) + \Delta_2^2 \exp\left(-\frac{t}{\tau_2}\right) + \Delta_3^2 \exp\left(-\frac{t}{\tau_3}\right) + \Delta_4^2 \exp\left(-\frac{t}{\tau_4}\right) \cos(\omega_4 t).$$

Here, we used these parameters, which are collected in Table 1(b). This $M(t)$ does not include a Gaussian component, because its exponential terms reflect the high frequency vibrational modes, which dominate the protein inertial motions, usually reflected by a fast Gaussian component.

For simplicity, we use the same $M(t)$ for the native, unfolded and intermediate conformers. We are therefore assuming the same homogeneous dynamics (solvent dynamics and chromophore vibrations) for the three conformers. This assumption is reasonable because although the conformational state has a measurable effect on the porphyrin vibrations and the solvent

contributions, their impacts on the lineshape are small relative to the change in the magnitude of inhomogeneous broadening. However, since the APS of the native and unfolded protein are different, we add different inhomogeneous terms when calculating $g(t)$. To reproduce the APS, we incorporate $\Delta_{in} = 150, 260$ and 200 cm^{-1} , respectively, for native, intermediate and unfolded conformers. The intermediate is attributed the largest inhomogeneous broadening because its absorption spectrum (shown in Fig. 3e) has the widest FWHM. The peak extinction coefficient of the three conformers is slightly different (Fig. 3e). Although it would be more accurate to weight the contributions to the optical response accordingly, we neglect this small effect. There is some uncertainty in the exact values of ω_{eg} , the 0–0 transition energy, however, as they are not experimentally available. Nevertheless, as ω_{eg} should always be at the red side of the absorption maximum, we chose the following values for the simulation: 420, 426 and 427 nm for the unfolded, intermediate and native conformers, respectively. For each GuHCl concentration and folding model (two- or three-state), the population is calculated based on the global fitting parameters ($C_{m(1,2)}$ and $m_{(1,2)}$) shown earlier. Then, the corresponding optical response function is a sum of these conformers, with the response of each conformer weighted according to its population. For example, since the midpoint sample consists of a nearly 1:1 mixture of native and unfolded forms in the two-state model, its total optical response function is a sum of native and unfolded samples, with equal contributions from the two conformers. Finally, the calculation includes the effects of finite pulse duration (45 fs), bandwidth, and center wavelength, as implemented in previous work [19].

Fig. 4 shows the simulated 3PEPS for native, unfolded states and midpoint in the two-state model. At $T = 0$, the three states have similar initial peak shifts: ~ 25 fs. As mentioned earlier, the initial peak shift is inversely proportional to the homogenous line broadening, which is characterized by $M(t)$. Actually the simulated initial peak shifts are slightly greater than the experimental values ~ 20 fs. The spectrum of vibrational mode-specific displacements for Zn-cyt are not available, thus we do not attempt to quantitatively reproduce the homogeneous lineshape. With our model $M(t)$, the homogeneous broadening is smaller than the actual value, so the simulated initial peak shifts are expected to be larger than measured values. The simulation reproduces the prominent oscillation in the experimental data, and the three samples have identical peak and trough positions, although the fluctuations are not as well-resolved in the experiment due to limitations in the signal to noise. The APS from the simulations are 4.2, 5.7 and 7.8 fs for native, unfolded and midpoint states, respectively. These values match the experiment, within the measurement error (Table 1(a)), which indicates that this folding process can be explained by a two-state folding model.

We calculated the asymptotic peak shift as a function of GuHCl concentrations for both two- and three-state models (Fig. 5a). In three-state model, the APS increases from 0 to 2.5 M GuHCl, then decreases and levels off at GuHCl concentrations above 3.5 M where the population is constant. Interestingly, for GuHCl concentrations below 2.5 M, the difference between two and three-states is insignificant. From our experimental data, we cannot rule out the possibility of a three-state model, because this model predicts an APS at 2.5 M GuHCl concentration that differs from the experimental value by only 0.3 fs. This difference is within the error of our measurement. However, the predictions of the two models differ significantly in the 2.5–3.5 M GuHCl range. Therefore, a titration measurement in this concentration window could be useful for distinguishing between two and three-state models, since the APS values differ significantly, and exceed the error of the experiment (typically less than ± 0.5 fs) [19].

Next, we investigated the effect of laser wavelength on the APS of the midpoint sample by varying the laser wavelength both experimentally and numerically at a GuHCl concentration of 2.5 M, as shown in Fig. 5b. At this GuHCl concentration, the sample consists of a 1:1 mixture of native and unfolded conformers for two-state model, or an approximately 1:1 mixture of

intermediate and unfolded conformers for the three-state model, as shown in Fig. 2c and d. For the three-state model, the APS increases when the laser wavelength is tuned to 428 nm. This result is physically reasonable because the ω_{eg} of intermediate is assumed to be 426 nm. Therefore, pump laser bandwidth is tuned to preferentially probe this conformer, which has the largest inhomogeneous broadening conformer. For the same reason, the APS reaches its maximum at 420 nm for two-state model. The measured APS at a few wavelengths is also shown in this figure. Although data at longer wavelengths would be more conclusive, the available data are more consistent with the values and the trend of the two-state unfolding model.

5. Discussion

In the two and three-state models discussed above, we used the same value of homogeneous broadening for each conformer, but differing amounts of inhomogeneous broadening, to simulate our experimental results for the midpoint state. Within the two-state model, the maximal inhomogeneous broadening of the midpoint state is caused by the mixture of native and unfolded conformers. Although three-state folding with largest inhomogeneous broadening for the intermediate conformer could also explain these results, it is not consistent with our wavelength dependent measurement. From the perspective of the Soret-band lineshape, Zn-cyt c in GuHCl behaves as a two-state system.

Our findings are consistent with the results of Gray, Winkler and coworkers who used triplet state decay kinetics to probe the unfolding of Zn-cyt c [31]. The rate of triplet decay in the presence or absence of a quencher ($\text{Ru}(\text{NH}_3)_6^{2+}$) reflects the hydration and solvent accessibility of the Zn-porphyrin. The triplet lifetime decreases about 10-fold in the unfolded species. Comparison of these rates to those for a small tryptic fragment of Zn-cyt c indicates the porphyrin is solvent exposed in the GuHCl unfolded state. At the midpoint GuHCl concentration, they observe biphasic triplet decay kinetics: a fast component corresponding to an extended, partially solvent-exposed state and a slow component attributable to a compact, relatively solvent-inaccessible state. These experiments suggest that Zn-cyt c is a two-state unfold and that the intermediate simply consists of a mixture of the native and unfolded states.

Bren and coworkers used fluorescence resonance energy transfer (FRET) to investigate the folding of Zn-cyt c [32]. They chose a three-state model to interpret the data, in which the equilibrium constants are different from our three-state. This result may seem contradictory to our results, as we interpret the midpoint state as a 1:1 mixture of native and unfolded conformers. However, femtosecond nonlinear spectroscopy and FRET results are complementary, because FRET reflects global conformational change, whereas femtosecond nonlinear spectroscopy is dependent upon the local porphyrin environment. One possible consistent picture is that the chemical environment of the porphyrin has only two states, while the protein may have a larger number of global states during denaturation.

In summary, we report 3PEPS measurements of the equilibrium unfolding of Zn-cyt c. We observe the largest asymptotic peak shift at the midpoint denaturant concentration and attribute this to a bimodal Inhomogeneous Distribution Function (IDF) arising from a mixture of two conformations as opposed to the widely assumed Gaussian IDF [33,34]. In fact, many proteins, such as receptors, are thought to exist in two or more families of functional conformational states, each of which may have conformational diversity. Since different conformations are usually not optically or chemically distinguishable, a simple bi- or multi-Gaussian model may still not be applicable. Therefore, for a spectroscopically complicated system such as a protein, it would be worthwhile to develop a method for measuring not just the width, but also the shape of the IDF.

Acknowledgments

This work was supported by the National Science Foundation DBI0454763, the Physics Frontier Center at JILA (PHY0551010), and by NIH/CU Biophysics Training Grant (T32 GM-065103) (to Z.S.). R.J. is a staff member in the Quantum Physics Division of NIST.

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Fig. 1. Peak shift traces for native, midpoint (2.5 M GuHCl) and unfolded (4.5 M GuHCl) equilibrium states of Zn-cyt c, and nonlinear least squares fits (solid lines). Inset: UV-vis spectra.

Fig. 2.
Absorbance changes of Soret band of Zn-cyt c as a function of GuHCl concentration.

Fig. 3.

Unfolding transition of Zn-cyt c measured by Soret band absorption changes. Plots (a) and (b) show the experimentally measured changes in optical density as a function of GuHCl concentration for different wavelengths including the fits by two- (a) and three-state (b) unfolding models (solid lines). Parts (c) and (d) show the calculated population of the conformers versus GuHCl concentration for two- and three-state unfolding models, respectively. Part (e) is the computed absorbance spectra of the native, intermediate and unfolded conformers for the three-state model. Note that the two-state model includes just the native and unfolded conformers.

Fig. 4.
Simulated 3PEPS traces for native, midpoint and unfolded Zn-cyt c.

Fig. 5. Comparison of two- and three-state models. (a) Simulated asymptotic peak shift of Zn-cyt c with varying GuHCl concentration. (b) Simulated asymptotic peak shift for the midpoint state of Zn-cyt c (2.5 M GuHCl) with varying laser wavelength.

Table 1

(a) Fitting parameters of peak shift data to function for the multiple exponential decay components and asymptotic peak shift. (b) Parameters for homogeneous broadening used for the 3PEPS simulations.

(a)					
Sample	τ_i (fs)	A_i (fs)	$\tau(\infty)$ (fs)	ω_0 (cm ⁻¹)	ϕ_0 (rad)
Native	53	11.7	4.2		
	3027	1.8			
Unfolded	58	6.9		157.5	9.3
	32	9.6	5.7		
	5153	1.7			
Midpoint	50	5.9		160.8	9.4
	34	12.8	7.75		
	56	6.1		157	
(b)					
Δ (cm ⁻¹)	τ (fs)	ω (cm ⁻¹)	ϕ (rad)		
226.3	55				
66.7	320				
59.7	7450				
97.14	415	216	0		