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The Two Dimensional Vibrational Echo of a Nitrile Probe of the Villin HP35 Protein

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Abstract

2D IR spectroscopy was used to probe the hydrophobic core structure of the 35-residue Villin headpiece subdomain, HP35, by monitoring the C≡N vibrational stretching band of a cyano substituted phenylalanine (Phe). The presence of two humps in the vibrational frequency distribution in the folded equilibrium state is revealed. They represent two states that exchange more slowly than ca. 10 ps. The two CN stretch mode peak frequencies (and their equilibrium populations) are 2228.7 (44%) and 2234.5 cm^{-1} (56%). The two CN modes have different frequency-frequency correlation times of 7.4 ps and 1.6 ps respectively. These results suggest that the population with the higher frequency CN group is partly exposed whereas the other CN mode experiences a hydrophobic like environment.

Keywords

Nitrile probe; Two dimensional infrared spectroscopy; Villin headpiece; protein structural heterogeneity; cyanophenylalanine

The increasing availability of molecular dynamics simulations of protein structural fluctuations and of folding pathways [1–4] raises interesting questions about how the implied, detailed, microscopic predictions, which often involve ultrafast structural fluctuations, can be tested by real time experiments that monitor events that occur faster than the rate determining steps of conventional kinetics experiments.[5] Different peptide conformations necessarily have different dispositions of backbone and side chains so that they have different vibrational spectra. Two dimensional infrared spectroscopy (2D IR) [6–9] is a method of choice for examining protein structural constraints and their fluctuations over a broad time range. The peptide backbone amide groups have coupling parameters that are in principle deducible from these multidimensional infrared methods. The parameters of 2D IR in turn relate to the “instantaneous” backbone structure. The inverse bandwidth of commonly occurring vibrational transitions sets a time scale of a few picoseconds, such that conformational exchanges would have to occur faster than this to be motionally averaged.[10] Thus all the major structures anticipated in peptide conformational dynamics are expected to be spectrally distinct in 2D IR and with better resolution than FTIR.

The dynamics within the vibrational frequency distribution of a mode, often referred to as spectral diffusion, signifies the time dependent structure of the immediate environment of the mode. This information is contained in the frequency-frequency correlation functions obtained from 2D IR.[9] Measurements of these correlations over a wide range of times reveal

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the dynamics local to an amide as well as those associated with the coupling between modes on different sections of the secondary structure.[9,11] These concepts apply equally well to the vibrational modes of probes that are engineered into the backbone or side chains of the peptides. The probes could be non-perturbative isotopologues or chemically modified residues as in the present paper.

Nitrile groups have been very useful as vibrational probes of biological systems [12–15] using FTIR methods which measure frequencies and bandwidths. However 2D IR experiments can provide better spectral discrimination of underlying components along with the relaxations of the frequency distributions at each point in the 2D spectral plane.[9,10] In addition 2D IR measures separately the homogeneous and inhomogeneous contributions to the spectrum and it establishes whether the inhomogeneous part changes with time. For some model systems the 2D IR spectra have shown that the frequency relaxation of the CN mode is strongly solvent dependent and that a novel perspective on the structures and motions of solvent molecules nearby to the CN group can be obtained.[16,17] In the present work the 2D IR method is applied to a small protein HP35, the headpiece subdomain of the actin-binding Villin.[18] The frequencies and frequency fluctuations of a nitrile group substituted on a phenylalanine (Phe58), which is part of the hydrophobic cluster, is used to probe the protein in its equilibrium folded state distribution at pH 4.9 and 300K.

Based on high resolution NMR [18] and X-ray diffraction [19] the HP35 folded structure is a 3 α-helix bundle, where the two short helices H1 (residues 44–51) and H2 (residues 54–60) and the longer helix H3(residues 64–74) confine a hydrophobic core (PDBID: 1VII). This hydrophobic region has been associated with the stability and folding of HP35 [20], possibly involving the aromatic-aromatic π-π interactions between phenylalanines, although recent experiments have indicated that the backbone H-bonding interactions within the H3-helix are key to the stability of the folded structure [21] and that only one of the turns is well structured in the folding transition state.[22] The present paper is focused on the equilibrium structure distribution and on the fluctuations within the hydrophobic core region of the folded state of Villin HP35 by means of 2D IR spectroscopy on a chemically modified HP35 (hereafter referred to as HP35-P) where a C≡N vibrational probe (Phe_{CN}) is synthetically incorporated into the sidechain of Phe58 on H2-helix.

The experimental FTIR spectrum of HP35-P in H_2O presented in Fig. 1(a) (black dotted, solid line) shows a single peak corresponding to the C≡N stretching band centered at 2233.7 cm^{-1} and having full width at half maximum (FWHM) of 13.6 cm^{-1}. The band is very slightly asymmetric suggesting that there might be two transitions separated by less than the bandwidth (see below). When constrained to consist of two Voigt profiles the linear FTIR spectrum would fit to two peaks separated by 5 cm^{-1} with a peak area ratio of 0.53±0.20 (see Figure 1(a)). However it is not possible to establish the presence or the characteristics of these two bands from the linear FTIR spectrum of HP35-P alone.

The 2D IR vibrational photon echo experiment is described in the Methods section. The spectra of HP35-P displayed in Figure 2 are basically plots of the pump coherence frequency ω_τ versus the probe coherence frequency ω_t for various waiting times T.

Figures 2(a–e) each show two regions with opposite signs corresponding to the v=0→1 transition (red) and to the v=1→2 transition (blue) regions. At T= 0 ps (Fig. 2(a)) the 2D spectrum is most elongated along the diagonal of the 2D axes, but it broadens and becomes less elliptically shaped as T increases. The variation with T of the peak value of the projection of the 2D spectra onto the ω_t axis yields the population relaxation time, T_1 =4.2 ± 0.2 ps, which is in the range of the published values of T_1 for model nitrile systems. [13,16,23] The average separation between the peaks of the differently signed regions is ca. 25 cm^{-1}

which is consistent with known anharmonicities for CN stretch modes.[13,16,17,24] The lower frequency part of the v=0→1 region in ω_τ axis is narrower along the probe coherence axis (ω_t) than is the higher frequency part. The separation between peaks of positive and negative signal along ω_t increases with decreasing ω_τ again suggesting that there are two populations having different CN mode frequencies and relaxation parameters. The presence of the two peaks is most easily seen by inspection of the traces of the 2D IR spectra along the diagonal line $\omega_\tau = \omega_t$ (diagonal slice) which show double peaked distributions (Figure 1(b)), which will be referred to as modes A (lower frequency peak) and B (higher frequency peak). The frequencies, deduced from numerical fitting, are 2228.7 ± 1.3 cm^{-1} (mode A) and 2234.5 ± 0.7 cm^{-1} (mode B). The complete absence at any time delay T of cross peaks at the points $\{\omega_\tau = \omega_B, \omega_t = \omega_A\}$ or $\{\omega_\tau = \omega_A, \omega_t = \omega_B\}$ in the 2D IR spectrum indicates that there is no coupling between modes A and B nor is there any exchange between the conformations they represent on the experimental time scale. The data show that the exchange time A↔B must be longer than ca. 10 ps.

The 2D IR spectra were simulated using a sum of all non-linear response functions that describe the photon echo signal detected in the $-k_1+k_2+k_3$ direction.[25] The diagonal signal at a given point in the two dimensional space $\{\omega_\tau, \omega_t\}$ has a T dependent shape that is proportional to $\mu^4 c_{eq} \sum_p R_p(\omega_\tau, \omega_t; T)$ where μ is the transition dipole moment, c_{eq} is the equilibrium population and R_p $(\omega_\tau, \omega_t; T)$ is the double Fourier transform of the p^{th} Liouville pathway response function contributing to the signal at that point.[9] The spectral diffusion is contained in the T dependence of the spectral shape defined by the sum of the responses. The following frequency-frequency correlation function having four parameters T_2^*, Δ, τ_c *and* σ for each of the two modes was incorporated into the response functions:

$$C(t) = \delta(t)/T_2^* + \Delta^2 e^{-t/\tau_c} + \sigma^2. \quad (1)$$

The first term is the homogeneous relaxation T_2^*, the second term is a Kubo function that accounts for the spectral diffusion depending on the parameters Δ and τ_c chosen for each of the modes, and the third term, σ, represents any static inhomogeneity. A linear least square fit of the 2D spectra was performed with the frequencies of the two transitions fixed to the values found from the fit of diagonal traces, and assuming the T_1 times are the same for the two modes (4.2 ps). The results of the fit of 2D IR spectra are displayed in Figure 2 (right panel) and the parameters found are discussed below. The ratio of the signal strengths from the A and B components is $c_A\mu_A^2/c_B\mu_B^2$ in the linear spectrum and $c_A\mu_A^4/c_B\mu_B^4$ in the 2D IR spectrum.[16] These ratios are found to be 0.53 (linear) and 0.24±0.09 (nonlinear) which immediately leads to the results $|\mu_A/\mu_B|$ 0.67 ± 0.18 and $c_A/(c_A + c_B)$ 0.44 ± 0.11. The frequencies of the A and B modes and the ratio of the transition dipoles are quite similar to results for the C≡N of benzonitrile in THF (A) and H_2O (B), where the dipole ratio is 0.74 ± 0.14 and the frequencies are 2230.5 cm^{-1} and 2235 cm^{-1}.[17] The results for HP35-P are therefore consistent with the C≡N in state A sensing a predominantly hydrophobic environment: It has a lower frequency and a smaller transition dipole than B. Furthermore its frequency relaxation is much slower than that for the population B, which is more similar to that of CN in water. The static inhomogeneity, σ, computes to be the same (0.28 ± 0.16 ps^{-1}) for both states while the homogeneous relaxation time, T_2^*, though not so well determined, is found to be longer for state A (>20ps) than state B (4.8 ± 4.3 ps). The static inhomogeneity corresponds to the remnant correlation from processes that dephase the vibration more slowly than the time interval over which the signal is observed, ca. 10 ps. Slow protein backbone fluctuations must be responsible for this residual part of the correlation function which is present in both A and B states.

The magnitude of the Kubo term at T=0 is somewhat larger for state B (0.79 ± 0.16 ps^{-1}) than A (0.66 ± 0.14). The slower τ_c of 7.4 ± 4.4 ps for mode A implies the C≡N senses a slowly responding medium such as might be expected from the motion of the side chains and backbone in the folded state. The response resembles that seen in hydrophobic solvents such as THF. Previous work for C≡N probes in the nonnucleoside inhibitor TMC278 complexed with HIV-1 reverse transcriptase [13] revealed a comparable value for τ_c of 7.1 ps, for a CN embedded in a hydrophobic pocket of the protein. Thus we identify the slower dynamics with fluctuations in the hydrophobic core of HP35-P. The much faster correlation time of 1.6 ± 0.6 ps for mode B is more characteristic of the time scales found for amide carbonyl groups in water and is twice as fast as bulk water itself [26,27] and therefore is very likely to be caused by the making and breaking of hydrogen bonds between the CN and water molecules.

The anharmonicities of the A and B modes are 27.7 ± 1.3 and 23.5 ± 0.7 cm^{-1} respectively: both values are consistent with the anharmonicity of the C≡N stretch mode in different media.[13,16,17,24] A Morse oscillator model implies that the relationship between the fundamental frequency and anharmonicity is $\omega_{10}=\omega_H-\Delta$, where ω_{10} and ω_H are the fundamental and harmonic frequencies, and Δ is the anharmonicity. The similarity of the differences of anharmonicities (4.2 cm^{-1}) and fundamental frequencies (5.8 cm^{-1}) for A and B modes are consistent with the nitrile groups being Morse oscillators with the two states A and B having the same harmonic frequency. Such differences could arise from the H-bonding of CN with, for example, water. The 2D spectral shapes(Figure 2) resemble those of a simple oscillator having a significant frequency dependence of the diagonal anharmonicity.[28] However, in the present case the shapes could not be reproduced unless two oscillators were incorporated into the fit.

An essential caveat is whether the CN substitution significantly perturbs the HP35 structure so that the behavior observed is not a characteristic of the native structural ensemble. A simulation including the CN in the peptide may provide an answer to this question. According to the NMR structure (PDBID: 1VII) the C-H bonds from residue Lys70 in the H3-helix would require to undergo some relaxation to make room for the CN group. In as much as the probe CN is unlikely to change the secondary structure of the protein, it is concluded that the results expose two tertiary structures of HP35. Two states of the native “folded” distribution of HP35 were also identified from experiments involving triplet energy transfer between chemically inserted aromatic probes.[29] These authors reported 75% of a compact folded state and 25% of a so-called molten globule form. The latter was shown to have slightly increased solvent accessible surface area from opening of the H3 helix wall of the hydrophobic pocket. However in our experiments the dominant population (B) is the one having the CN more accessible to solvent water. It should be noted that there are examples of other proteins where nitrile substitutions did not perturb the structure.[30,31]

The 2D IR spectroscopy results require that the villin HP35 protein subdomain sensed by a chemically modified phenylalanine (i.e., cyanophenylalanine) is conformationally heterogeneous. The conformational distribution has two peaks which we have modeled as two CN modes A and B. The measurements of the correlations of the CN frequency fluctuations show that in sub-ensemble A the C≡N probe is buried in the hydrophobic core of the protein. The sub-ensemble with the higher CN frequency, B, has parameters that are more similar to CN groups associated with some water molecules. This in turn would require that B correspond to a less compact structure where the C≡N probe can frequently access groups with which it can make and break hydrogen bonds. Since there are no cross peaks in the 2D IR signals of the pair of transitions A and B, the exchange between them must be slower than ca. 10 ps but faster than the time scale of ca. 1 ms, of the NMR experiments and faster than the 100 ns kinetic component of protein folding/unfolding seen

in a linear IR T-jump experiment [32] that did not see these conformational states. The sub-ensemble environments A and B are distinguished in the fits to the 2D IR spectra by the differences in their C≡N stretching transition frequencies, transition dipole moments and the correlation times of their frequency-frequency autocorrelation functions. The combination of linear and nonlinear infrared methods provides a compelling case for the interpretation given and the results should represent a novel test of simulations of the protein dynamics and of the associated CN vibrational frequency relaxation.[33,34]

EXPERIMENTAL METHODS

Materials

The current studies were carried out with an HP35 mutant with sequence: L_{42}SDEDFKAVF-GMTRSAFANL-PLWKQQNLKK-EKGLF$_{76}$, where the Phe58 was substituted by a *p*-cyanophenylalanine; synthesized using standard FMOC chemistry on a PS3 (Protein Technologies) peptide synthesizer. HP35-P was then purified to homogeneity by reverse-phase HPLC. The mass was verified using matrix assisted laser desorption ionization spectroscopy (Ultraflex, Bruker). The CD vs. Temperature melting curve shows that the protein is in the folded state (ca. 90%) under the experimental conditions. The sample was checked for aggregation in the Amide-I region in neat D_2O, where non β-signatures were observed. The experiments were done with H_2O as the solvent and with a protein concentration of ca. 7mM. A 60mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution was used to stabilize the solution at pH 4.9.

Two dimensional spectra

The laser system has been fully described [35], but in brief the 2D IR spectra were obtained by exciting the sample with three pulses 1,2 and 3 (wave-vectors k_1, k_2 and k_3) having energy of ca. 400 nJ, pulsewidths of 80 fs, and center frequency of 2235 cm^{-1}. The signal in the phase matching direction $-k_1+k_2+k_3$ was detected by heterodyning it with a local oscillator pulse and focusing it at the entrance slit of the monochromator (with a groove density 100 lines/mm), and then detecting with a 64 element HgCdTe array detector. All the fields were chosen for parallel polarization (zzzz). The time interval between pulses 1 and 2 is the coherence time t; that between the pulses 2 and 3 the waiting or population time, T; and that between pulse 3 and the detected signal is t, the detection interval. The rephasing and nonrephasing sequences correspond to k_1 arriving earlier or later than k_2, respectively. All of the 2D IR spectra were obtained by sampling the coherence time from −4 ps to 4 ps in steps of 2 fs. To obtain the absorptive spectra, rephasing and nonrephasing 2D frequency spectra were added. The detailed data processing methods have been described previously.[35]

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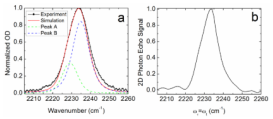

Figure 1.

FTIR and diagonal slice of the 2D spectrum of HP35-P in H_2O. a) The curve with black circles corresponds to the normalized experimental FTIR; the solid red curve is the Fourier transform of the linear response function constructed with parameters from the fit of the 2D spectra as discussed in the text; the blue and green dashed curves are two fitted components of the simulated spectrum. b) Signal magnitude along a diagonal line ($\omega_\tau = \omega_t$) of the experimental 2D spectrum at T=0.5 ps (see Figure 2(b)) showing a double-humped distribution.

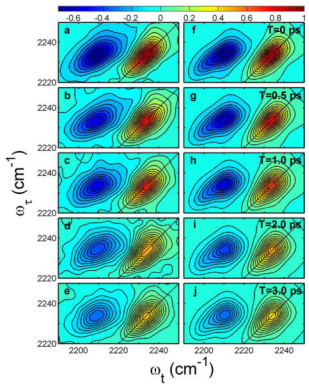

Figure 2.

Real absorptive 2D IR spectra for HP35-P. Experimental spectra, left panel Figs.(a–e), suggest that the transition at ca. 2235 cm^{-1} is composed of a double peaked frequency distribution. The spectra in the right panels (f–j) correspond to those computed from the response functions as described in the text for two nearby transitions. The color bar at the top of the Figure applies to all the panels. The indicated waiting times (T) apply to each row.