Vol 14 No 12, December 2005
1009-1963/2005/14(12)/2484-07

Chinese Physics

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Nanosecond-time-resolved infrared spectroscopic study of fast relaxation kinetics of protein folding by means of laser-induced temperature-jump*

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(Received 6 July 2005; revised manuscript received 8 September 2005)

Elucidating the initial kinetics of folding pathways is critical to the understanding of the protein folding mechanism. Transient infrared spectroscopy has proved a powerful tool to probe the folding kinetics. Herein we report the construction of a nanosecond laser-induced temperature-jump (T-jump) technique coupled to a nanosecond time-resolved transient mid-infrared (mid-IR) spectrometer system capable of investigating the protein folding kinetics with a temporal resolution of 50 ns after deconvolution of the instrumental response function. The mid-IR source is a liquid N_2 cooled CO laser covering a spectral range of 5.0μm (2000 cm^{-1}) $\sim$ 6.5μm (1540 cm^{-1}). The heating pulse was generated by a high pressure H_2 Raman shifter at wavelength of 1.9μm. The maximum temperature-jump could reach as high as 26$\pm$1°C. The fast folding/unfolding dynamics of cytochrome C was investigated by the constructed system, providing an example.

Keywords: protein folding, sub-microsecond, laser-induced temperature-jump, transient infrared spectrum, dynamics, cytochrome C

PACC: 3320E, 0570, 0720

1. Introduction

The question of how peptides efficiently and reliably achieve their native structure is one of the most intriguing problems. In the 1960s Anfinson proposed that the native structure of a protein is determined by its amino acid sequence: that is, the sequence of amino acids in a peptide chain determines its folding pattern.[1] This is known as the central rule which has proven correct for most cases, though there are a few exceptions. The fundamental question is, therefore, how a polypeptide chain of astronomically possible conformations finds its unique native biologically active structure in a finite time. The problem has been known as the Levinthal paradox which states: if a protein is made up of a polypeptide chain of 100 residues, a random search of all conformations would require 10^{27} years.[2] Obviously this is not true. Generally, the folding time of proteins can be less than several minutes, so it is clear that evolution has found an effective solution to this folding problem. Levinthal then proposed that there existed specific pathways for folding.[3] On these specific pathways, the protein molecules would pass through well-defined partially structured states. Up until now, knowledge regarding the fast folding dynamics is mainly from theoretical modulations such as molecular dynamic simulations. In contrast, the progress in experiments lags behind.

The experimental study of the early events in protein folding requires a method for the initiation of the fast folding/unfolding process. There are three commonly used approaches: i.e., stopped-flow, laser-induced temperature-jump (T-jump) and laser-

*Project supported by the National Natural Science Foundation of China (Grant No 20373088) and Program for innovation group (Grant No 60321002).

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induced photo-dissociation technique. However, the time resolution of the stopped-flow technique is limited by its typical mixing "dead time" in a range of sub-microsecond to microseconds, during which the faster folding process has already been completed for many proteins. Though the time resolution of the laser-induced chemical reaction technique can reach as fast as picoseconds, it is limited to the study of specific proteins or polypeptides where photochemical reactions can initiate an unfolding/folding process. Among those fast folding initiation methods, the T-jump method seems to be the most versatile because it is a nonspecific method and a temperature jump can be realized in a period of time as short as several tens of picoseconds. Another advantage is that it does not need the introduction of extraneous reagents into the folding system.[4,5] By judicious choice of temperature and solution conditions, it is possible to shift the equilibrium between the native and denatured forms to be poised at any point, and then increase in temperature to perturb the equilibrium toward a more folded or more unfolded position.

The use of fast T-jump method to displace chemical equilibria and measure relaxation rates was pioneered by Eigen and de Mayer,[6] they employed the rapid capacitance discharge to produce Joule heating in conducting solutions. This approach is limited to the microsecond time scale. In the past decades, the technique of using temperature jump produced by absorption of short laser pulses to study rapid reactions has been developed. Early attempt was to utilize dyes to absorb Q-switched pulses from ruby and Nd:glass lasers, providing radiation at 694 and 1060 nm, respectively, and succeeded in generating a temperature jump of $\sim$4 °C.[7,8] Temperature jump produced by direct absorption of near-infrared pulses by water was first described by Turner *et al.*[9,10] They generated the heating pulse with a duration of 14–18ns at 1.41μm by Raman-shifting the Nd:YAG fundamental in liquid N_2. Williams *et al*[11] extended this method by using methane gas as the Raman shift medium producing the heating pulse at 1.54μm, and applied the T-jump technique to study the salt effects on duplex formation of dGCATGC. The laser-induced T-jump technique has recently been applied to the folding/unfolding kinetic study of the peptides and proteins by several groups. Williams *et al*[12] used a Nd:YAG-pumped dye laser to generate a $\sim$7ns near infrared pulse at 2.0μm by difference frequency mixing in a $LiNbO_3$ crystal. By using infrared detection to monitor the amide I infrared absorption (1630–1660 cm^{-1}), they observed a relaxation time of 160 ns for the decrease in the average helical content of a 21-residue alanine-based peptide after an 18°C temperature jump. This technique has also been used to study the unfolding of apomyoglobin.[13] Huang *et al*[14] used Raman-shifting of H_2 gas to generate 1.9μm for helix-coil transition of an alanine based peptide. Because vibrational relaxation in water takes place on the picosecond timescale,[15,16] and the diffusion of heat out of the interaction volume takes several milliseconds, the T-jump technique provides a dynamical range of nearly 10^6 in the temporal range.

Herein we described a fast T-jump apparatus capable of acquiring kinetic relaxation transients via time-resolved mid-infrared (mid-IR) absorption spectroscopy over a time range from sub-microseconds to milliseconds. The apparatus provides a time-resolution of about 50 ns after the deconvolution of the instrumental response [17] and a sensitivity about 10^{-4} in the infrared absorbance difference. The maximum T-jump achieved is approximately 26°C. We also report the results of the T-jump/time-resolved transient infrared spectroscopic study of the fast folding/unfolding dynamics of cytochrome C (Cyt.C) in D_2O.

2. Experimental

The laser-induced T-jump/time-resolved mid-IR transient difference absorbance spectrometer is shown schematically in Fig.1. The 1.9μm heating pulse was generated via Raman shifting with the fundamental output 1064 nm of a Nd:YAG laser (Spectrum Physics Lab 170, repetition rate:10 Hz, pulse width: 10 ns) in a Raman cell filled with H_2 at a pressure of 5.2×10^6Pa. The Nd:YAG laser was protected from optical damage caused by amplification of back-reflected pump light and backward stimulated Brillouin scattering by using an optical isolator consisting of a thin film polarizer and a quarter-wave plate. Limited by the length of the Raman cell (a stainless steel cylinder fitted with two $\Phi15\times10$ mm quartz plates as the windows) and the pressure of the H_2, the maximum achievable output power of the 1.9μm pulse is about 25$\pm$0.5 mJ.[18] Liquid N_2 cooled CW CO laser (made in Dalian University of Technology) was employed as the mid-IR probe beam, which was tunable from 5.0μm (2000 cm^{-1}) to 6.5μm (1540 cm^{-1}) by changing the grating angle using a computer. The output power was about 8mW/cm^2 and the stability was around$\pm$2%. The

1.9μm heating beam and the infrared probe beam were overlapped at the focused point, the sizes of the beams being 1.5 mm and 650μm respectively. The angle between the two beams was about 7.6°. The solution sample was sealed between two CaF_2 plates separated by a 56μm thick teflon spacer. To reduce the error arising from the replacement of the sample cell and that of the reference solvent, a two–compartment cell was used with one compartment for the sample and the other for the reference solution without protein. A monochrometer and Ge filter were used to eliminate the scattering light while Ge filter also played a role of adjusting the intensity of the mid-IR probe beam. The T-jump signal was detected by a photovoltaic HgCdTe detector (100MHz, Shanghai Institute of Technical Physics, CAS). Then the signal was amplified by a preamplifier (SR440, DC~300MHz, Stanford Research Systems INC.) and filtered by a low-pass filter (DC~20MHz). Finally, the signal was displayed by a digital oscilloscope (Tektronix TDS500D) and data were transferred to a personal computer.

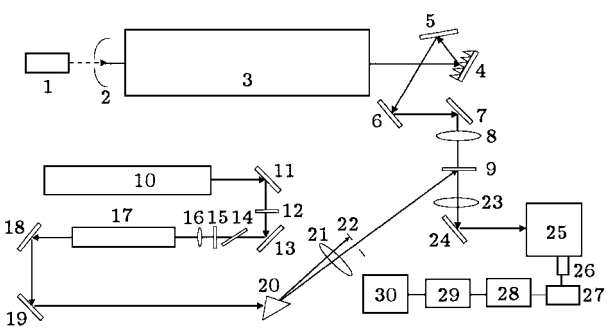


Fig.1. Schematic of laser-induced temperature-jump, time-resolved infrared apparatus. 1: Indicator He–Ne laser; 2: concave reflective mirror with a pinhole; 3: vacuum chamber of CO laser with refrigerant liquid N_2; 4: blazed grating; 5, 6, 7 and 24: gold coating reflective mirror; 8 and 23: CaF_2 lens; 9: sample cell; 10: Nd:YAG laser; 11 and 13: 1064nm reflective mirror; 12: $1/2\lambda$ wave plate; 14: thin-film polarizer; 15: $1/4\lambda$ wave plate; 16 and 21: lens; 17: Raman-shifting cell; 18 and 19: 1.9μm reflective mirror; 20: Pellin–Broca prism; 22: a pinhole; 25: monochrometer; 26: HgCdTe detector; 27: pre-amplifier; 28: low-pass filter; 29: oscilloscope; 30: PC.

3. Results

The overtone absorption band of water at 1.54μm or D_2O at 1.9μm is the most frequently used excitation wavelength for generating a T-jump in H_2O/D_2O solution. The mid-IR transmission spectra of H_2O and D_2O are presented in Fig.2. For infrared absorption studies of proteins in solution, D_2O is often used instead of H_2O in order to shift the strong water absorption band which normally overlaps with the amide I band (from 1600–1700 cm^{-1}). The 1.9μm laser energy is absorbed by a small volume of D_2O at the focus by vibrational excitation, and the temperature of the volume of D_2O reaches its maximum value in approximately 20 ns (twice the full width at half-maximum (FWHM) of the pulse width). The magnitude of the T-jump is calibrated using the change of D_2O absorption with temperature, which acts as an internal thermometer in the frequency range of 1630–1700 cm^{-1}.[12]

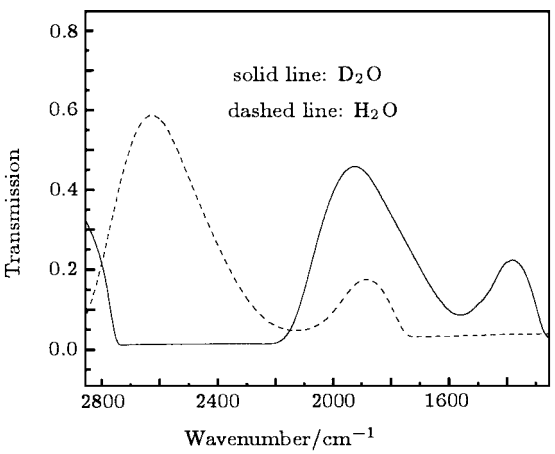


Fig.2. Mid-infrared transmission spectra of H_2O and D_2O.

Temperature-dependent Fourier transform infrared (FTIR) absorbance spectra of D_2O were obtained using an ABB-BOMEN FTIR spectrometer. D_2O was purchased from Beijing chemical factory with a purity of 99.8% and sealed under a N_2 atmosphere in a 56μm thick infrared cell and the temperature was controlled to an accuracy within ±0.25°C using a thermostat (Harrick Corp.). Difference spectra were generated by subtracting the infrared spectra acquired at the indicated temperature by that of room temperature at 26°C as shown in Fig.3. The plot of the corresponding infrared absorbance difference of D_2O against the temperature yielded almost a linear relation between the absorbance change and the corresponding temperature at each measured wavelength as shown in Fig.4. Figure 4 presents a typical linear decrease of the optical density change (ΔOD) with the increase of the temperature at 1650 cm^{-1}, the linear relation holds in a temperature range from 30 to 80°C with a slope of 2.68×10^{-3} ΔOD/°C. Figure 5 gives a series of transient infrared absorbance difference signal of various T-jump magnitude in D_2O, where the temperature jump ΔT was deduced from the calibration shown in Fig.4, which indicates that one percent of ΔOD corresponds to a ΔT of about 3.73°C. T-jump

relaxation trace of D_2O is shown in Fig.6 with a decay time constant of ~6ms.

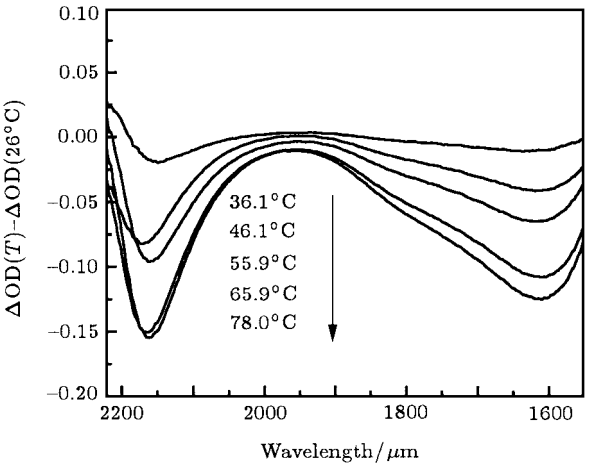


Fig.3. Difference FTIR spectra of D2O at the indicated temperatures with respect to that at 26°C.

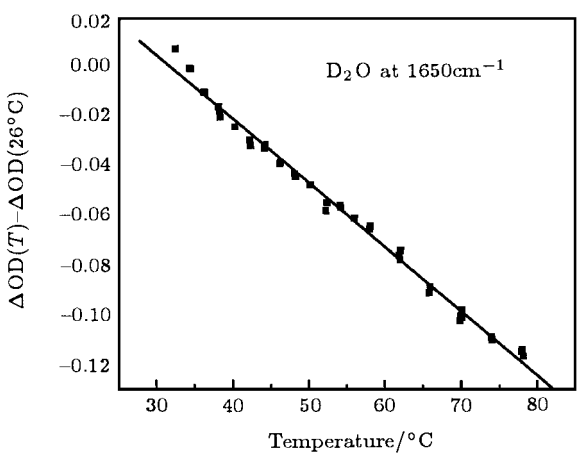


Fig.4. The relative optical density (ΔOD) of D_2O at 1650 cm^{-1} as a function of temperature.

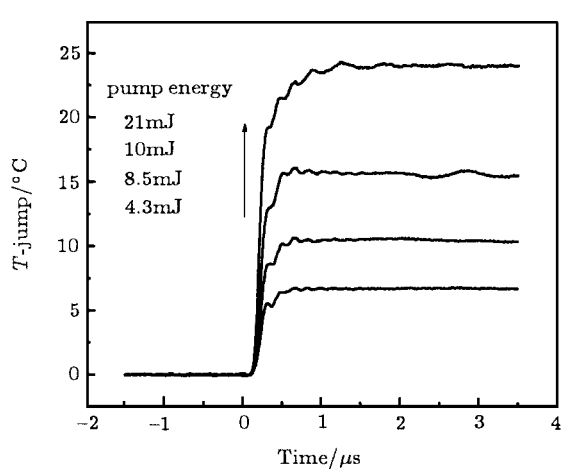


Fig.5. The signal of T-jump in D_2O at 1650 cm^{-1} for different pumping energy.

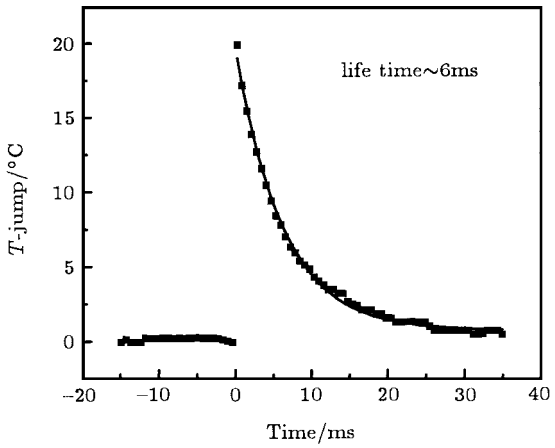


Fig.6. The relaxation kinetics of T-jump signal in D_2O at 1650 cm^{-1}.

With the constructed laser-induced T-jump/time-resolved mid-IR transient difference absorbance spectrometer, the T-jump induced unfolding/folding kinetics of Cyt.C can be investigated. We choose horse heart Cyt.C for the first application of the device since Cyt.C has a compact folding intermediate with a secondary structure content. Cyt.C is a single domain protein of 104 amino acids. A covalently attached heme group is surrounded by three major helices (designated as the N-terminal, C-terminal and 60s helices), and an iron atom is coordinated by His 18 and Met 80.[19] Cyt.C (C-7752) from horse heart was purchased from Sigma without further purification. For both steady-state and time-resolved IR experiments, the samples were dissolved in D_2O at a pD of 6–7.5 and incubated for 12h at 30°C to exchange marginally protected amide protons, the final concentration was 10–13mg/mL.

The FTIR absorbance spectra of Cyt.C in D_2O (C=1mM, 56μm in path length) at different temperature are shown in Fig.7(a). The amide I' absorption envelope shifts to a low frequency by 3–5 cm^{-1} as the temperature increases and it can be explained as the H/D exchange behaviour upon heating.[20] Due to considerable overlap of the structural components in the raw absorbance spectra, identifying the equilibrium changes of the various structural components is difficult. Resolution enhancement of the steady-state FTIR spectra (at 46°C) via second-derivative analysis reveals five major amide I' subcomponents at 1612, 1632, 1650, 1674 and 1684 cm^{-1} (Fig.7(b)). The peak in the amide II' region at about 1566 cm^{-1} originates predominantly from the peptide N-H bending and C-N stretching.[20] Previous studies of model peptide and protein in D_2O reveal that the absorption band near

$1612\,cm^{-1}$ arises from the protein aggregation[21–23] while α-helix generally exhibits an amide I' absorbance near 1632 and $1650\,cm^{-1}$.[24] $1674\,cm^{-1}$is assigned to β-sheet structures in Cyt.C,[20,25] and $1684\,cm^{-1}$to β-turn structures.[20] Figure 7(c) shows the difference absorption spectrum generated by subtracting the FTIR spectrum acquired at the indicated temperature from that at 18.9°C, which reveals the dynamical change of these various subcomponents upon thermal denaturation. As the temperature increases, the contents of α-helix and β-sheet in Cyt. C are reduced, while the component corresponding to the aggregated protein [26] increases. The transient difference spectrum at 4μs after the T-jump (ΔT=22°C) is shown in Fig.8 at the room temperature (23°C). With a T-jump of 22°C, the bleaching at 1634 and 1650 cm^{-1} indicate the unfolding of the solvated helix and the buried helix respectively.[24] The peaks at 1600 and $1608\,cm^{-1}$ are assumed to arise mainly from the aggregated form, and the sharp peak at $1644cm^{-1}$ can be attributed to the increase of the unordered structure.[21] Figure 9 shows the kinetic trace after the T-jump probed at $1644\,cm^{-1}$ and $1650\,cm^{-1}$. The inset shows the transient absorption signals of the D_2O reference and the Cyt.C in D_2O solution at $1645\,cm^{-1}$. We are able to distinguish the transient absorption changes of the Cyt.C despite the fact that most of the absorption changes are due to a very fast change in the broad mid-IR absorption background of D_2O with temperature. Consequently, extreme care must be taken to subtract the large D_2O contribution from the transient absorption data. The subtraction error is minimized in these experiments by using the two-compartment cell having a sample and a reference compartments which are precisely matched in path-length and temperature as described in the previous section. Fitting the kinetics with a biexponential function convolved with an instrument response function, the bleach kinetics at $1650\,cm^{-1}$ (α-helix) has a fast phase (90%) with a rising time constant of 120 ns and a slow phase (10%) of 2.3μs. The instrument response function was determined by fitting the T-jump kinetics of D_2O alone convoluted with a Gaussian type of instrument response function (FWHM=150ns).[27] In contrast, the absorption signal of unordered structure at $1645\,cm^{-1}$ contains a 20% fast component with a time constant of 170ns followed by a 80% slow component with a rising time constant of 2μs. We attribute the fast phase kinetics at $1650\,cm^{-1}$ to the relaxation process of α-helix and the slow phase kinetics to the process of helix-coil interaction. The fast and slow kinetics at $1645\,cm^{-1}$

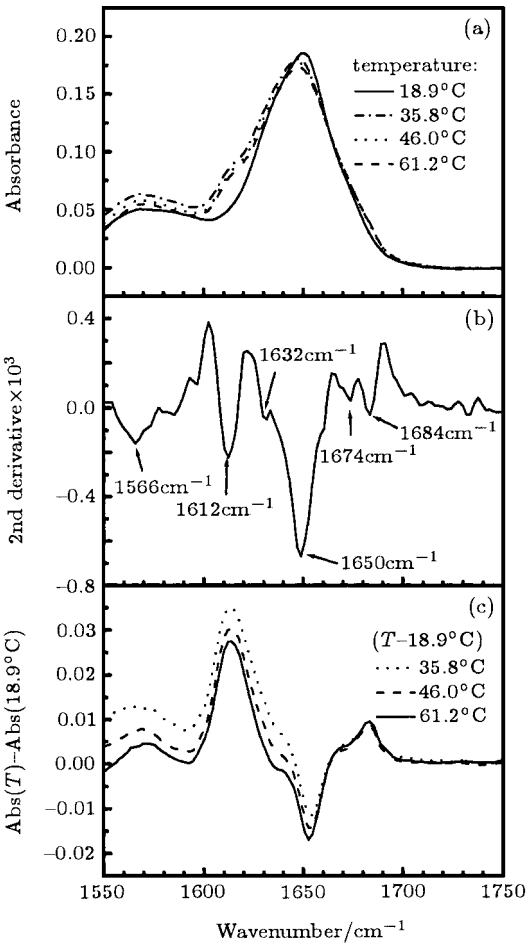


Fig.7. (a) FTIR absorbance spectra of Cyt.C in D_2O buffer, 1mM pD=7 at different temperatures. (b) Second-derivative spectra at 46°C. (c) FTIR difference spectra of Cyt.C. The difference spectra are obtained by subtracting the spectrum at 8.9°C from the spectrum at each temperature.

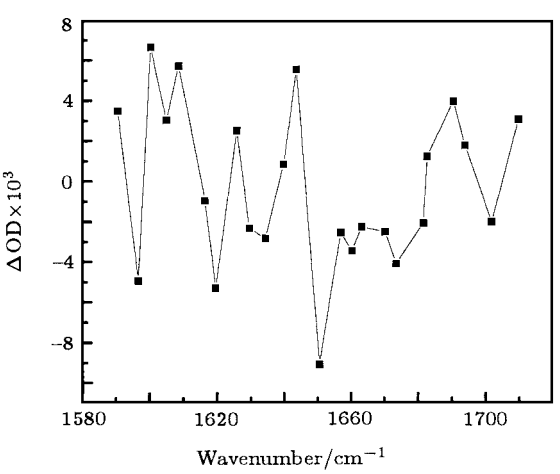


Fig.8. Time-resolved IR spectrum at 4μs (— ■ —) after a T-jump of 22°C.

may result from the folding/unfolding process of α-helix and β-sheet structures and from helix-coil interaction and/or aggregation structures mentioned above.

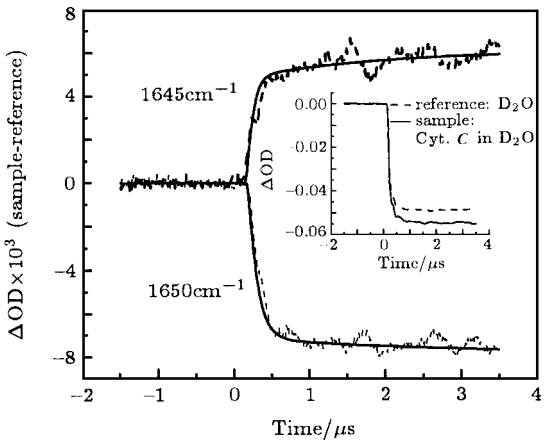


Fig.9. Change in optical density (ΔOD) at 1645 cm^{-1} of D_2O (reference, inset dashed line) and Cyt.C in D_2O (sample, inset solid line).Kinetic IR response for a T-jump from 23 to 55 °C at 1645cm^{-1} and 1650 cm^{-1} (dashed line). The background D_2O response has been subtracted from these data. The fit to a biexponential function convolved with the instrument response is shown as a solid line.

4. Discussion

To determine the magnitude of the T-jump, we choose 1650 cm^{-1} as the measuring wavelength in the range of 1630–1700 cm^{-1} where D_2O has a linear change in absorption with the temperature at each wavelength. The magnitude of the T-jump has been examined at a number of wavelengths at 1630, 1650 and 1670 cm^{-1} in D_2O buffer solution. The results yield an average uncertainty of about ± 1°C, similar to the results of Timothy's.[12] Furthermore, the relaxation time of the T-jump signal could be controlled by varying the thickness of the sample. For D_2O, the T-jump relaxation time constant are 10.7, 7 and 6ms corresponding to a spacer thickness of 100, 70 and 56μm respectively. In order to achieve a high signal-to-noise ratio (S/N), a 56μm teflon spacer was chosen.[28]

The essential characteristic of the T-jump is that it perturbs the equilibrium position of the ensemble of possible states faster than the chemical processes that lead to the equilibrium. This is the fundamental characteristic of relaxation methods.[6] After the T-jump, the system "relaxes" to the new equilibrium point. In general, the kinetics observed thereafter involves both the forward and backward reactions. For a simple two-state kinetics, the observed relaxation rate is the sum of the forward and backward reaction rates, and each can be determined separately if the equilibrium constant is known.[29] The T-jump method is essentially a relaxation approach with which both refolding and unfolding processes may be studied no matter in which direction the equilibrium shifts.

5. Conclusion

We have built a laser-induced temperature-jump setup to initiate the folding and unfolding processes on sub-microsecond timescale, and employed the time-resolved infrared absorption spectroscopy to probe the subsequent protein unfolding/folding kinetics. The apparatus can efficiently change the temperature of the sample within the limit of the pulse duration for a nanosecond heating pulse to initiate a fast protein unfolding/folding process. The constructed laser-induced T-jump coupled to a time-resolved mid-IR transient difference absorbance spectrometer has been successfully applied to the investigation of the folding/unfolding process of the Cyt.C, which reveals some typical fast folding/unfolding events of the protein subcomponents. The system provides a powerful tool for studying the early protein folding events and would be helpful to a quantitative and predictive understanding of the factors that control or determine folding pathways, the stable structures, as well as thermally and kinetically accessible conformational sub-states in the conformational ensemble of the protein.

Acknowledgment

We thank Professor Q. X. Yu for his kind technical support.[30]

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