Multifrequency calorimetry of the folding/unfolding transition of cytochrome c

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ABSTRACT The folding-unfolding transition of Fe(III) cytochrome c has been studied with the new technique of multifrequency calorimetry. Multifrequency calorimetry is aimed at measuring directly the dynamics of the energetic events that take place during a thermally induced transition by measuring the frequency dispersion of the heat capacity. This is done by modulating the folding/unfolding equilibrium using a variable frequency, small oscillatory temperature perturbation (~0.05–0.1°C) centered at the equilibrium temperature of the system. Fe(III) cytochrome c at pH 4 undergoes a fully reversible folding/unfolding transition centered at 67.7°C and characterized by an enthalpy change of 81 kcal/mol and heat capacity difference between unfolded and folded states of 0.9 kcal/K*mol. By measuring the temperature dependence of the frequency dispersion of the heat capacity in the frequency range of 0.1–1 Hz it has been possible to examine the time regime of the enthalpic events associated with the transition. The multifrequency calorimetry results indicate that ~85% of the excess heat capacity associated with the folding/unfolding transition relaxes with a single relaxation time of 326 ± 68 ms at the midpoint of the transition region. This is the first time that the time regime in which heat is absorbed and released during protein folding/unfolding has been measured.

INTRODUCTION

Throughout the years the thermodynamics of protein folding/unfolding has been studied by many researchers. These studies have focused primarily on the energetics of the transition between the folded and unfolded states and the mechanisms of structural stabilization of proteins. The thermodynamic analysis of proteins reached its maturity several years ago with the development of high-sensitivity differential scanning calorimetry (Privalov, 1979). Before this development, the experimental approach relied mostly on a variety of physical observables (primarily spectroscopic) whose properties were sensitive to the folding/unfolding process. This approach was limited to few protein systems because the energetics of folding/unfolding cannot be determined by noncalorimetric techniques unless the transition involves only two states (Freire and Biltonen, 1978; Privalov, 1982). The development of high-sensitivity differential scanning calorimetry allowed for the first time a direct measurement of the folding/unfolding energetics of proteins in a model independent fashion.

Contrary to the thermodynamic analysis, the kinetic analysis of protein folding has relied primarily on a variety of observables that report time-dependent changes of some system property as the system evolves from the folded to the unfolded state or vice versa. For many proteins, different physical observables report distinct numbers of relaxation processes and amplitudes. To date these relaxation processes have not been correlated to the dynamics of the enthalpic events associated with a folding/unfolding transition. This paper reports the first direct measurement of the time evolution of the energetic parameters of protein folding/unfolding using the new technique of multifrequency calorimetry.

The folding/unfolding transition of cytochrome c has been studied before from both thermodynamic and kinetic points of view. A number of observables has been used to monitor the kinetics of the temperature, pH, or guanidine induced unfolding or refolding: heme absorption at a number of different wavelengths (Ikai et al., 1973; Tsong, 1973; Nall et al., 1988), tryptophan fluorescence (Nall et al., 1988, Roder et al., 1988), circular dichroism (Kawajima et al., 1987), differential scanning calorimetry (Privalov, 1979), and hydrogen-deuterium exchange (Roder et al., 1988). Whereas the thermodynamic studies indicate that the folding/unfolding transition is a two-state transition (i.e., no thermodynamically stable partially folded states are significantly populated during the folding/unfolding process), the kinetic studies have revealed the existence of at least three different relaxation processes occurring on the time scales of microseconds, milliseconds and seconds. A complete understanding of the folding/unfolding transition requires a way of assigning the enthalpic contributions of

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each of the measured relaxation processes to the overall transition enthalpy.

The principal motivation behind the development of the multifrequency calorimeter has been precisely the development of an experimental way of measuring directly the dynamics of the energetic changes that take place during protein folding/unfolding. The multifrequency calorimeter accomplishes that goal by generating a small temperature oscillation of variable frequency that modulates the folding/unfolding equilibrium. In this paper we describe the application of this technique to study the dynamics of the energetic changes associated with the folding/unfolding transition of Fe(III) cytochrome *c*.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. (St Louis, MO). The stated purity was 99%, and the protein was used without further purification. It was stored dessicated and frozen until used. The calorimetric studies of the folding/unfolding transition were performed at pH 4 in 50 mM sodium acetate buffer. The intially prepared protein solutions were dialyzed at 4°C for at least 16 h against 300 vol of buffer. Protein concentrations were measured spectrophotometrically using an extinction coefficient of 29.5 mM^{-1} cm^{-1} at 550 nm for the reduced form of cytochrome *c* (Watt and Sturtevant, 1969). All experiments described in this paper were performed with Fe(III) cytochrome *c*.

Conventional high-sensitivity differential scanning calorimetry experiments were performed on a MC2 instrument (MicroCal, Inc., Amherst, MA) interfaced to a microcomputer system using a model DT2801 (Data Translation Inc., Natick, MA) data acquisition board (Myers et al., 1986). The analysis of the calorimetric data was performed with software developed in this laboratory.

Multifrequency calorimetry experiments were performed on a new version of the instrument originally developed in this laboratory (Mayorga et al., 1988; Freire et al., 1990). A block diagram of this instrument is shown in Fig. 1. This second generation instrument incorporates the following new features: (*a*) a high-precision solid-state temperature control mechanism; (*b*) a Fourier series wave generator connected to a battery of miniature Peltier elements to provide the temperature excitation. This feature allows the use of up to 10 different excitation frequencies simultaneously; (*c*) a battery of miniature semiconductor thermopiles on the measuring face of the sample cell is used to measure the system response to the excitation temperature wave. The output of the measuring thermopiles is amplified and the signal fed to a spectrum analyzer (model 35660 A; Hewlett-Packard Co., Palo Alto, CA). The spectrum analyzer performs the Fourier transform of the data in real time and provides the amplitude and phase angle of the reponse function.

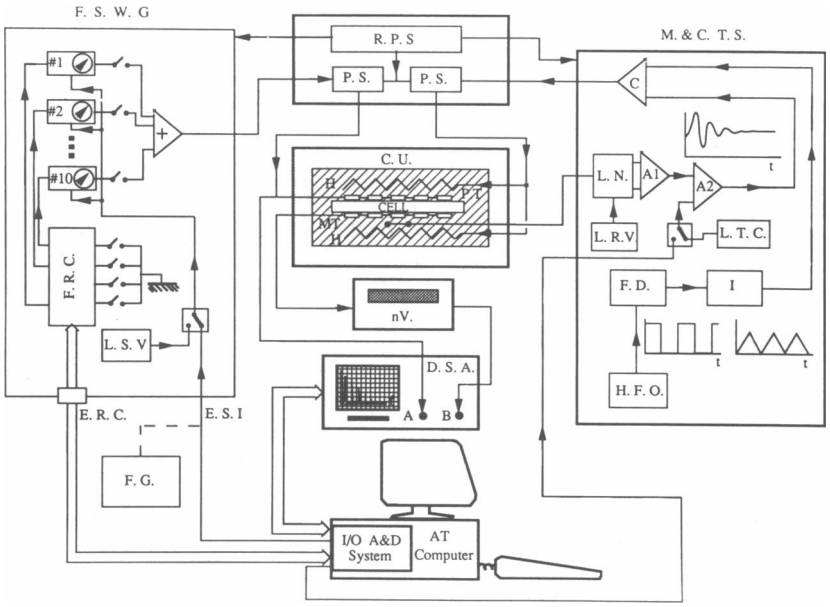

FIGURE 1 Schematic diagram of the multifrequency calorimeter used in the experiments reported in this paper. In this figure the abbreviations refer to the following components of the instrument: (*i*) The calorimetric unit (C.U.) where the cell and associated electronics are located (measurement and perturbation thermopiles (MT and PT) as well as temperature transducers for feedback temperature control). (*ii*) Fourier Series Wave Generator (F.S.W.G.) used to generate the periodic temperature perturbation and containing 10 precision sine wave generators connected to a summing amplifier, a frequency range controller (F.R.C.), a local sweeping voltage (L.S.V), an external range controller (E.R.C.), and an external sweeping input (E.S.I.) (*iii*) Measurement and Control Temperature System (M. & C.T.S.) for thermostating the calorimeter including a thermistor response linearizing network (L.N.), precision amplifiers (A1 & A2), high frequency oscillator (H.F.O.), frequency divider (F.D.), integrator (I.), and local reference voltage (L.R.V.). (*iv*) The response signal is amplified by a nanovoltmeter (nV) connected to a dynamic signal analyzer (D.S.A.) interfaced to a microcomputer system for real time Fourier analysis.

Multifrequency calorimetry experiments were performed according to the following protocol. A cytochrome *c* solution was thoroughly degassed at room temperature and then loaded into the calorimeter cell. The system was allowed to equilibrate at a preselected temperature. Once thermal equilibrium was established, the steady-state temperature response of the system to a periodic temperature perturbation was measured over the frequency bandwidth 0.1–1 Hz. The temperature perturbation consists of a superposition of 6–10 sinusoidal waves of different frequencies and is applied on the perturbation face of the calorimeter cell. The temperature oscillations measured on the opposite face of the calorimeter cell, after traveling through the protein solution, constitute the system response. The equilibrium temperature settings were then increased and the process repeated. All experiments were done from low to high temperature, and covered the range 35 to 85°C. The amplitude of the perturbation temperature oscillation was in the range of 0.05–0.1°C. The multifrequency calorimeter experiments were performed using cytochrome *c* concentrations of 60–70 mg/ml.

RESULTS

Theory

The theory of multifrequency calorimetry has been presented elsewhere (Freire et al., 1990). Briefly, the heat capacity at constant pressure, C_p, is directly proportional to the mean square amplitude of the equilibrium fluctuations in the enthalpy of a system:

$$C_p = [\overline{H^2(t)} - \overline{H}^2]/RT^2, \quad (1)$$

where the overbars indicate the long time average. This result can be derived, assuming the system to be ergodic, from Einstein fluctuation theory (Landau and Lifshitz, 1980) or directly from the partition function (Hill, 1986).

Because the thermodynamic heat capacity can be defined as a long time average, it contains no information about the dynamic characteristics of the enthalpy fluctuations. That information is contained in the normalized autocorrelation function,

$$[\overline{H(t + t')H(t)} - \overline{H}^2]/[\overline{H^2(t)} - \overline{H}^2], \quad (2)$$

the measurement of which is a central concern for the spectral resolution of enthalpy fluctuations. Linear response theory and the fluctuation-dissipation theorem indicate that the enthalpy autocorrelation function is accessible, in the frequency domain, from stationary temperature perturbation experiments, the relevant observable being a frequency-dependent heat capacity (Landau and Lifshitz, 1980; Birge, 1986; Freire, 1989; Freire et al., 1990).

A protein existing in thermodynamic equilibrium between folded and unfolded conformations undergoes dynamic structural fluctuations between the various states that are accessible to it under the externally imposed conditions. According to the fluctuation-dissipation theorem (Landau and Lifshitz, 1980) the dynamics of those fluctuations are characterized by the same parameters that characterize the return to equilibrium after a small external perturbation. The natural frequencies of the normal modes of the spontaneous fluctuations are related to the frequencies at which the system can optimally absorb thermal energy (which can be substantial, in view of the excess heat capacity these systems display). The relaxation times for the normal modes are given as the inverses of the natural frequencies, and they characterize the frequency-dependence of the apparent excess heat capacity.

Thermodynamic analysis of cytochrome c folding/unfolding

Before study by multifrequency calorimetry, the folding/unfolding transition of the cytochrome *c* samples was characterized by high-sensitivity differential scanning calorimetry. The measured excess heat capacity curve of cytochrome *c* at pH 4.0 is shown in Fig. 2. As shown in the figure, the experimental curve can be well represented by a two-state transition mechanism within the limits of experimental error. In fact, deviations between experimental and theoretical curves predicted by the equations corresponding to a two-state transition mechanism (Freire, 1989) are never $> 1\%$, in agreement with previous conclusions (Privalov and Khechinashvilli, 1974). According to the data in Fig. 2, the transition is characterized by a transition temperature (*Tm*) of 67.7°C, a transition enthalpy (ΔHcal) of 81.4 kcal/mol, and ΔCp of 0.9 kcal/K/mol. These values are in excellent agreement with those reported previously under similar experimental conditions (Privalov and Khechinashvilli, 1974).

For multifrequency calorimetry to be applicable, the

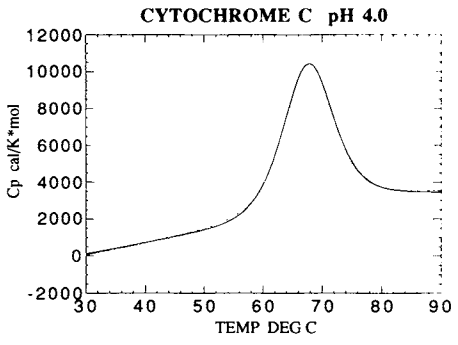

FIGURE 2 Temperature dependence of the thermodynamic excess heat capacity function of a solution of Fe(III) cytochrome *c* at pH 4, as measured by high-sensitivity differential scanning calorimetry. In this figure the dots represent the experimental data and the solid line represents the best fit of the data to a two-state transition mechanism as described in the text. The scanning rate of this experiment was 60 deg/h.

transition under investigation must be reversible. That is, it must satisfy the principle of microscopic reversibility at equilibrium, at all temperatures where measurements are to be made. The reversibility of the cytochrome *c* transition under the conditions used in the multifrequency calorimetry experiments was checked by performing repeated DSC scans on the same samples. These experiments are illustrated in Fig. 3 in which the results of two consecutive scans of the same sample are presented. Under those conditions the reversibility of the transition is better than 97%.

Multifrequency calorimetry

The sample compartment in the multifrequency calorimeter consists of a disk-shaped gold cell of 6 cm diameter and variable thickness (0.1–1 mm). One face of the sample cell (the excitation face) is subject to a steady-state oscillatory temperature perturbation. This temperature disturbance propagates through the sample, creating a temperature field. The response of the system is measured on the opposite face of the cell (measuring face) and has the form

$$T(x,t) = T_o + \delta T \exp \cdot \left[-x\sqrt{\omega C_p^o/2k}\left\{1 + \left(\frac{1}{2C_p^o}\right)\sum \frac{C_{p,j}^{ex}(1+\omega\tau_j)}{1+(\omega\tau_j)^2}\right\}\right] \cdot \sin\left[\omega t - x\sqrt{\omega C_p^o/2k}\left\{1 + \left(\frac{1}{2C_p^o}\right)\sum \frac{C_{p,j}^{ex}(1-\omega\tau_j)}{1+(\omega\tau_j)^2}\right\}\right], \quad (3)$$

where T_o and δT are the equilibrium temperature and perturbation amplitude, respectively; x is the distance from the excitation face; $\omega = 2\pi\nu$ is the angular frequency of perturbation; k is the thermal conductivity; and C_p^o is the nonrelaxing heat capacity per unit volume.

The nonrelaxing heat capacity contains contributions arising from the solvent as well as the intrinsic heat capacity of the protein under study. The above equation has been written for the general case in which the thermodynamic excess heat capacity is composed of contributions from multiple relaxation modes, each characterized by a relaxation time, τ_j, and amplitude, $C_{p,j}^{ex}$, (Freire et al., 1990). As indicated by Eq. 3, the response signal is attenuated in amplitude and lags behind the excitation signal. The magnitude of these effects is proportional to the excess heat capacity of the system and associated relaxation times as shown in Eq. 3, thus providing a way of estimating its magnitude and relaxation kinetics. Because the magnitude of the amplitude attenuation is proportional to the heat capacity, it is predicted to be maximal at the transition temperature of the system under study. It is clear from Eq. 3 that the relaxation times and amplitudes of the excess heat capacity are available independently either from response amplitude or phase shift measurements. In these studies we have primarily relied on the analysis of the frequency dependence of the response amplitudes because, over the low-frequency bandwidth of these experiments they can be measured more accurately.

For each excitation frequency, the attenuation in the amplitude of the temperature oscillation on the measuring face of the calorimeter cell is maximal within the transition region. In general, a plot of the temperature dependence of the response amplitude will be characterized by the presence of a relative minimum at the temperature in which the heat capacity is maximal. This is illustrated in Fig. 4, using as an example the measured temperature dependence of the response amplitude (A) of cytochrome *c* at pH 4.0 obtained at an excitation frequency of 0.1 Hz. As shown in the figure the response amplitude shows a significant deflection at 67°C, the

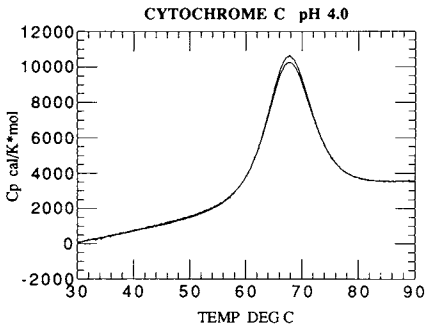

FIGURE 3 Results of two consecutive scans of a solution of Fe(III) cytochrome *c* at pH 4. As shown in the figure, the unfolding transition is ~97% reversible under the conditions of these experiments. The transition enthalpies differ by no more than 3%, and T_m and ΔC_p are unchanged.

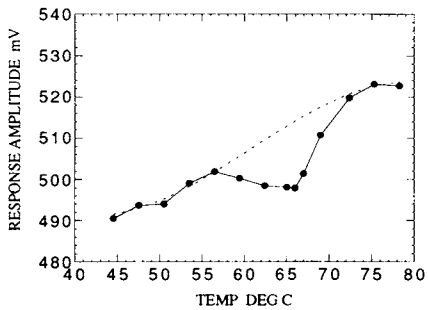

FIGURE 4 The MFC response amplitude of Fe(III) cytochrome *c*, measured at a perturbation frequency of 0.1 Hz and plotted with respect to temperature. The solid circles indicate the measured data, and the dotted line represents the estimated baseline data that would have been measured in the absence of any relaxing heat capacity.

location of the maximum in the thermodynamic excess heat capacity function (Figs. 2 and 3). In analogy with conventional differential scanning calorimetry the analysis of the data in Fig. 4 (and those obtained at other excitation frequencies) require estimation of a baseline; in this case, the response amplitude which would be observed if the sample did not undergo a transition. The procedure corresponds to baseline estimation in DSC, and is accomplished by linear or higher order interpolation between the sample response amplitudes measured at temperatures outside the transition region. This procedure is also illustrated in Fig. 4 in which a cubic polynomial baseline estimation is shown. Previously (Freire et al., 1990), we have shown that a convenient way of analyzing the data is by considering the ratio of the baseline response amplitude ($A°$) to the measured response amplitude (A). This ratio is given by the following equation:

$$A°/A = \exp\left[x\sqrt{\nu B/C_p^o}\sum \frac{C_{p,j}^{ex}(1 + 2\pi\nu\tau_j)}{1 + (2\pi\nu\tau_j)^2}\right] \quad (4)$$

and can be analyzed by a nonlinear least squares fitting algorithm to give the relaxation times and their corresponding contributions to the thermodynamic excess heat capacity function. In the above equation, B includes the constant terms in Eq. 3 and the instrument calibration constant (Freire et al., 1990). The quantity $A°/A$ has very important features as demonstrated for the case of a single relaxation process. In this case, the temperature-frequency surface defined by $A°/A$ has a maximum located at a temperature equal to Tm and at a frequency equal to $\frac{1}{2}\pi\tau$ or $(2\pi\tau)^{-1}$. This is due to the excess heat capacity being maximal at T_m and the system being excited at its natural frequency. In general the relaxation modes of the frequency dependent heat capacity are characterized by the amplitudes, $C_{p,j}^{ex}$ and the relaxation times τ_j, characteristic of the folding/unfolding transition of the protein.

Heat capacity dispersion of cytochrome c

The results of the multifrequency calorimetry experiments of cytochrome c at pH 4.0 are shown in Figs. 5 and 6. In Fig. 5 the ratio of the baseline response amplitude ($A°$) to the measured response amplitude (A) has been plotted as a function of temperature and perturbation frequency. This is the quantity described by Eq. 4. As shown in the figure, at each frequency the ration $A°/A$ has a maximum centered at 67°C, the transition midpoint, in agreement with the conventional DSC experiments shown in Figs. 2 and 3. Also, along the frequency axis the $A°/A$ surface shows a maximum at ~0.37–0.39 Hz. This is better illustrated in Fig. 6 where the

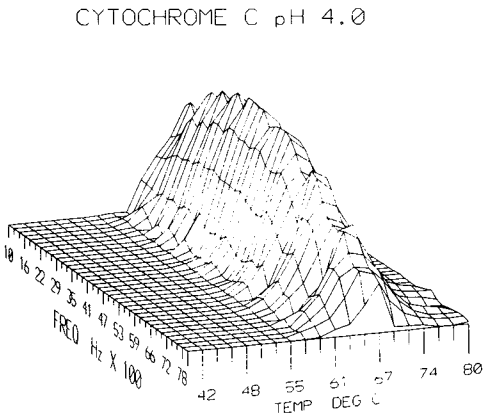

FIGURE 5 Plot of the response amplitude ratio $A°/A$ as a function of temperature and excitation frequency for Fe(III) cytochrome c at pH 4.0. The $A°/A$ ratios were calculated following the procedure described in Fig. 4.

temperature-frequency topographical map of the ratio $A°/A$ is shown. The location of this maximum is consistent with a relaxation time close to 400 ms.

Fig. 7 shows the results of the nonlinear least squares analysis of the values of $A°/A$ at the transition temperature. For the set of data shown in the figure, the characteristic relaxation time is equal to 380 ± 75 ms,

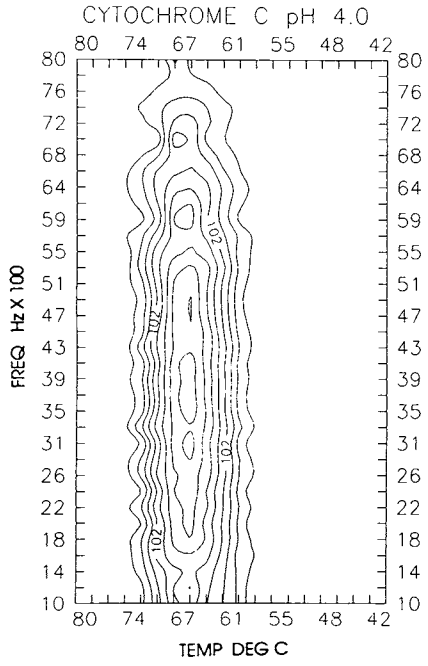

FIGURE 6 Topographic representation of the data in Fig. 5. The maximum of $A°/A$ occurs at a temperature of 67°C and at a frequency in the range of 0.35–0.5 Hz.

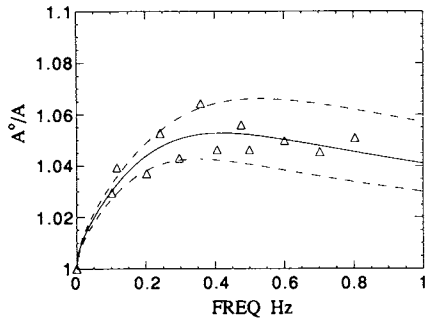

FIGURE 7 Frequency dependence of the response amplitude ratio $A°/A$ obtained at the transition temperature of Fe(III) cytochrome *c* at pH 4. The solid line represents the best nonlinear least squares fit of the data to a single relaxation process (see Eq. 4). The dotted lines indicate the one standard deviation confidence intervals for the amplitude and time constant which characterize this relaxation mode.

with a relaxation amplitude corresponding to ~85% of the equilibrium excess heat capacity at *Tm*. From a total of eight different experiments we obtained a mean relaxation time of 326 ± 68 ms with a relaxation amplitude corresponding to 85 ± 15% of the equilibrium excess heat capacity. The standard deviations include all cumulative errors associated with independent experiments performed with different protein samples. This error should not be confused with the intrinsic instrument precision. In fact, two consecutive measurements of the response amplitude of a given sample under the same conditions (excitation frequency and temperature) do not differ by more than 0.5% over the entire frequency range. Within the experimental error of these experiments, the goodness of fit could not be improved by increasing the number of relaxation processes from one to two. These results are consistent with the existence of a single predominant relaxation process that comprises most of the enthalpic events associated with the transition.

DISCUSSION

The kinetics of the folding/unfolding transition of cytochrome *c* at pH 4.0 has been investigated by the novel technique of multifrequency calorimetry. Working in the frequency range of 0.1 to 1 Hz, we have resolved a single relaxation of the excess heat capacity, characterized by a time constant of ~330 ms. This relaxation mode comprises most of the measured equilibrium excess heat capacity associated with the folding/unfolding transition.

In the past there have been several studies of the reversible unfolding kinetics of cytochrome *c*. Of particular relevance to our studies is the paper by Tsong (1973) because it contains a systematic characterization of the temperature dependence of the folding/unfolding kinetics. By monitoring heme absorbance at 408 nm, this author observed three relaxation processes. The fastest of these was only slightly temperature dependent, had a time constant of 40 μs and probably reflects only local changes in the heme group. The other two processes were characterized by relaxation times on the order of 10 and 300 ms in the temperature range of the multifrequency experiments, and showed a stronger temperature dependence, especially the slower process which exhibited an activation enthalpy of 18–25 kcal/mol.

In another study, Ridge et al. (1981) used GuHCl to trigger the folding and unfolding processes at pH 7.2. They observed two relevant kinetic events, with relaxation times of 200 ms and 19 s for refolding at 25°C. The slow folding and unfolding processes were characterized by an activation enthalpy of ~20 kcal/mol, from which a relaxation time of 300 ms is predicted at 65°C. These relaxation times are of the same order of magnitude as those found by Roder et al. (1988) for the refolding of the protein. Working at 10°C and using H-exchange labeling and proton NMR, they observed relaxation processes occurring on time scales of 20 ms, 200 ms, and 10 s. The first two processes involved protection of amide protons corresponding to the amino- and carboxy-terminal helices, and incipient formation of the helices between residues 65 and 75, respectively, as well as development of some global protection against exchange. The slowest process conferred the level of resistance to exchange typical of the native protein. The temperature dependence of these relaxation times was not investigated, preventing a direct comparison with other results.

According to the multifrequency calorimetry results, it is apparent that most of the enthalpic changes are associated with the slow kinetic phase observed by other techniques, or that this phase is rate limiting for the enthalpic changes associated with the transition. The observation that ~85% of the heat capacity relaxes in a single mode is consistent with the two-state character of the transition when studied under equilibrium conditions by high-sensitivity differential scanning calorimetry and other techniques.

The results presented in this paper constitute the first application of multifrequency calorimetry to a protein system. Multifrequency calorimetry is a new technique under continuous development in this laboratory. New generations of this instrument with improved sensitivity and broader frequency bandwidth are currently being implemented. These new instruments will permit a more thorough characterization of the dynamics of the energetic changes associated with protein folding/unfolding.

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