Structural Intermediates in Folding of Yeast Iso-2 Cytochrome *c*†

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ABSTRACT: The kinetic properties of the folding reactions of iso-2 cytochrome *c* from *Saccharomyces cerevisiae* have been investigated by stopped-flow and temperature-jump methods. Three different structural probes are compared: (1) absorbance changes in the visible reflecting changes in heme environment, (2) ultraviolet absorbance changes due to the exposure of aromatic groups to solvent, and (3) tryptophan fluorescence attributable principally to the average distance between the tryptophan residue (donor) and the heme (quencher). In addition, two probes either indicative of or correlated with function, ascorbic acid reducibility and the 695-nm absorbance band, have been used to monitor specifically the rate of formation of the native protein on refolding. The fastest phase observed (τ_3) has a measurable relative amplitude only when monitored by visible absorbance changes, suggesting that this reaction involves changes in heme environment in the absence of significant changes in the heme to tryptophan distance or in the extent to which aromatic groups are exposed to solvent. Different slow phases are observed when complete refolding is monitored by visible or ultraviolet absorbance (τ_1^a) as opposed to tryptophan fluorescence (τ_1^b), the fluorescence changes being complete on a time scale 4–8-fold faster than for absorbance. A mid-range kinetic phase (τ_2) is detected by all three structural probes. When ascorbic acid reducibility or 695-nm absorbance changes are used to monitor the rate of formation of the native protein, two phases are detected: τ_2 and τ_1^a. Taken together these results demonstrate that kinetic phase τ_1^b results in the formation of a structural intermediate in folding with fluorescence close to that of the native protein but with distinct absorbance properties.

The problem of how a polypeptide folds into a unique tertiary structure is both extremely simple and extraordinarily complex. Thermodynamic descriptions of folding are well established. Two-state mechanisms used to extract thermodynamic parameters from measurements of changes in physical properties on unfolding give numbers that agree well with those obtained from calorimetric measurements (Lapanje, 1978). Unfortunately, thermodynamics tells us nothing about the *process* of folding, but only about differences between the initial and final states.

The success of the two-state thermodynamic treatments of folding implies that protein folding is a highly cooperative process and thus that intermediates in this process will be difficult to isolate and characterize. Nevertheless, the kinetics of folding and unfolding of most proteins is a complex process indicating the presence, at least transiently, of multiple forms (Baldwin & Creighton, 1980). Two-state equilibrium behavior and multistate kinetic behavior may be compatible with each other if (1) the physical properties of the species observed kinetically are very close to those of the completely unfolded (U) or native (N) protein and therefore cannot be distinguished from the U and N species at equilibrium or (2) the intermediates are present in undetectably low concentrations at equilibrium and reach detectable levels only during a kinetic experiment. For ribonuclease A it appears that both of these possibilities play a role (Baldwin, 1980). There is strong evidence that some unfolded species which differ only by the cis or trans isomeric states of proline residues give rise to kinetically distinguishable species on refolding (Brandts et al., 1975; Stellwagen, 1979; Schmid & Baldwin, 1979). Thus under conditions where folding is blocked by proline isomerization, the unfolded state is composed of kinetically distinguishable but in most respects physically identical species. The second possibility is supported by other work with ribonuclease A which indicates that in some cases incorrect isomeric states of prolines retard but do not stop the folding process. This apparently leads to the transient formation of structured intermediates and nativelike species with incorrect proline isomeric states (Cook et al., 1979). Further work has shown that the structured intermediates which accumulate at slow steps in folding are highly hydrogen bonded, having a similar number of amide protons protected against exchange with solvent protons as are found in the secondary structure of the native protein (Schmid & Baldwin, 1979; Kim & Baldwin, 1980).

It is important to know whether or not the transient accumulation of structured intermediates in folding can be generalized to other proteins and in particular to proteins lacking the numerous disulfide bond cross-links found in ribonuclease A. Such intermediates may well play a critical part in folding by catalyzing intrinsically slow steps such as proline isomerization [see Levitt (1981)] or by drastically reducing the configurational space available to the polypeptide and thus directing the folding process to the native state. With this in mind, sensitive tests for the presence of structured intermediates have been applied to yeast iso-2 cytochrome *c* (iso-2).[1]

Previous results for iso-2 (Nall & Landers, 1981) have shown that the kinetic properties for the folding reactions for this protein are strikingly similar to those of the homologous protein from horse (Ikai et al., 1973; Tsong, 1976). Although the two proteins differ by 46% in amino acid sequence, they probably share the same tertiary structure. This suggests that the kinetic properties of folding reactions may be more closely associated with the kind of structures formed both as folding intermediates and as the final product of folding than with the particulars of the amino acid sequence. For example, the formation of α helices, reverse turns, and the packing of these

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[1] Abbreviations: iso-2, iso-2 cytochrome *c* from *Saccharomyces cerevisiae*; Gdn·HCl, guanidine hydrochloride; τ, time constant of a reaction (reciprocal of the apparent rate constant).

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elements of structure are processes that are expected to be similar for homologous proteins regardless of their particular differences in amino acid sequence. Processes and structures of these types become possible candidates for rate-limiting steps and for the kinds of intermediate structures formed in folding. Thus, the kinetic properties of folding reactions may reflect primarily the pathway of folding. Differences in amino acid sequence may be of secondary importance in that they alter the kinetic properties of folding only when they perturb the pathway of folding.

In the present work kinetic studies of the folding of iso-2 have been extended to include additional measurements of folding by fluorescence and absorbance in the stopped-flow and temperature-jump instrument. The results show that different structural probes detect different kinetic phases. If one defines structural intermediates as species present during folding with properties distinct from either the native or unfolded protein (Labhardt & Baldwin, 1979), then our results demonstrate the existence of at least two such intermediates. One of these intermediates is particularly interesting in that it seems to be a highly condensed structure with its aromatic groups at least partially exposed to solvent.

Materials and Methods

Stopped-flow measurements of time-dependent changes in fluorescence were made by mounting an EMI 9558Q photomultiplier tube over a Durrum fluorescence observation chamber at right angles to the optical path. A band-pass interference filter centered at 350 nm was placed between the observation chamber and the photomultiplier tube in order to remove scattered light at the excitation wavelength. A 200-W Hg–Xe arc lamp (Canrad-Hanovia) was used as a light source with the wavelength being selected by passing the light through a grating monochromator (Oriel Corp.) with slit settings that gave a band-pass of about 15 nm. A second photomultiplier (EMI 9558Q) was placed on the optical path so that absorbance changes could be monitored. For some of the experiments in which visible absorbance changes were monitored, a 100-W tungsten filament lamp was used as a light source.

For the relative amplitude and time constant data reported in Figure 3 and Table I each data point is an average of two or three independent measurements. Relative amplitudes were obtained by collecting both fast and slow phase data on a single mixing experiment by utilizing the split-time base mode of the transient recorder. In this way systematic errors in the relative amplitudes arising from small variations in mixing ratios from experiment to experiment were minimized. An exception is for total times in excess of 200 s where the split-time base mode could not be used since it was necessary to trigger the transient recorder externally. In this case relative amplitudes were obtained from separate mixing experiments which immediately followed one another.

In order to determine the fraction of the total equilibrium signal change accounted for in the kinetic experiments (Table I), it was necessary to measure the equilibrium signal change in the stopped flow. This was done by preparing solutions of iso-2 at a 6-fold lower concentration but which were otherwise identical with the protein solutions (before mixing) used in the kinetic experiments. These solutions were introduced into the stopped flow in order to estimate the signal levels before any folding (or unfolding) had occurred. For fluorescence Gdn·HCl-containing blanks were also introduced into the stopped flow in order to measure and correct for any background fluorescence from the Gdn·HCl.

Temperature-jump measurements of fluorescence changes were made on an Eigen-DeMaeyer temperature-jump instrument which was modified for fluorescence by mounting a photomultiplier tube near the sample cell at right angles to the optical path. The photomultiplier tube housing (EMI-Gemcon) contained an op-amp which increased signal gain and allowed the tube to be wired in a manner that gave adequate frequency response over the temperature-jump time range. Stray excitation light was removed with a 350-nm band-pass filter placed between the sample cell and the photomultiplier tube. For some of the temperature-jump experiments in which ultraviolet absorbance or fluorescence was monitored, the signal to noise was improved by computer averaging of 10–12 transients. For absorbance measurements the instrument was operated in a dual-beam mode, with the resulting photomultiplier signals being amplified by a log-ratio amplifier. Therefore, the signal changes are directly proportional to absorbance changes (10 mV = 10^{-3} absorbance).

For stopped-flow measurements the signal changes are proportional to transmittance. In analyzing the stopped-flow data it was assumed that the approximate relation

$$\Delta A(t) \sim \Delta I(t)/[2.303I(\infty)]$$

holds [where $\Delta A(t)$ and $\Delta I(t)$ are the differences in the absorbance and transmittance, respectively, at time t and at infinite time and where $I(\infty)$ is the total transmittance at infinite time]. For particularly large changes in transmittance, the total kinetic changes were converted to absorbance before calculation of the relative amplitudes.

Slow refolding was monitored spectrophotometrically in the range 200–800 nm on a Hewlett-Packard 8450 A UV–visible spectrophotometer. Folding was induced by mixing 0.5 mL of buffer with 0.1 mL of a protein solution containing 2.0 M Gdn·HCl by using an adder–mixer. The mixing dead time was about 5 s. Temperature was maintained at 20 ± 0.1 °C by circulating the thermostating liquid from a Haake circulating water bath through a thermojacketed cuvette.

Previously (Nall & Landers, 1981), samples of iso-2 cytochrome *c* were preunfolded by heating to 65 °C for 10 min in order to remove what are believed to be artifactual species which exhibit a very slow phase in unfolding. While the cause of these artifactual species is unknown, their presence is correlated with the following spectral properties for protein refolded from guanidine hydrochloride: (1) a slight loss in the intensity of the absorbance band at 695 nm and (2) the appearance of an often substantial absorbance band at about 650 nm. Heat treatment alone is not sufficient to permanently remove these species, and they have been observed to re-form slowly. In addition the samples used in the present study were preunfolded by exposure to greater than 3.5 M guanidine hydrochloride for 15 min or more. This seems to permanently remove the slow unfolding species as well as the aberrant spectral properties.

The methods of Ridge et al. (1981) were used to monitor the rate of formation of native cytochrome *c* by ascorbic acid reducibility. In monitoring fast refolding by reducibility (phase τ_2) ascorbic acid concentrations were maintained at high levels in the final conditions (0.44–0.88 M/L) to ensure that the unimolecular folding reactions were rate limiting. Controls in which native cytochrome *c* was mixed with ascorbic acid solutions indicated that the bimolecular rate of reduction was faster than the stopped-flow dead time (~3 ms) under the conditions employed to monitor refolding by reducibility. However, a slow kinetic event in the 1000-s time range was detected in these control experiments, which interferes with detection of slow refolding by reduction. The cause of this phase is unknown, but it could be due to residual protein which must isomerize before being able to react with ascorbic acid

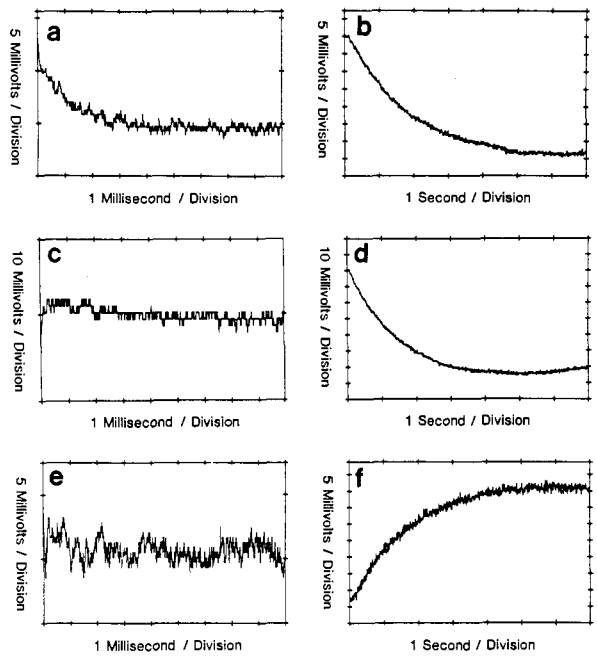

FIGURE 1: Temperature-jump measurements of unfolding from 15 to 20 °C in 1.4 M Gdn·HCl and 0.1 M sodium phosphate buffer, pH 7.2. Unfolding monitored by absorbance changes at 418 nm (10 mV = 10^{-3} absorbance): (a) τ_3 time range and (b) τ_2 time range. Unfolding monitored by absorbance changes at 287 nm: (c) τ_3 time range and (d) τ_2 time range. In (c) there is a slight curvature, suggesting that the amplitude of phase τ_3 may be near the noise level. Unfolding monitored by fluorescence changes: (e) the τ_3 time range and (f) the τ_2 time range. Phase τ_2 can be detected by both stopped-flow and temperature-jump measurements of unfolding, while phase τ_3 is slightly outside of the time range of the stopped flow and is detected only by temperature-jump measurements. There is some distortion of phase τ_2 at long times due to thermal cooling "schlieren" effects.

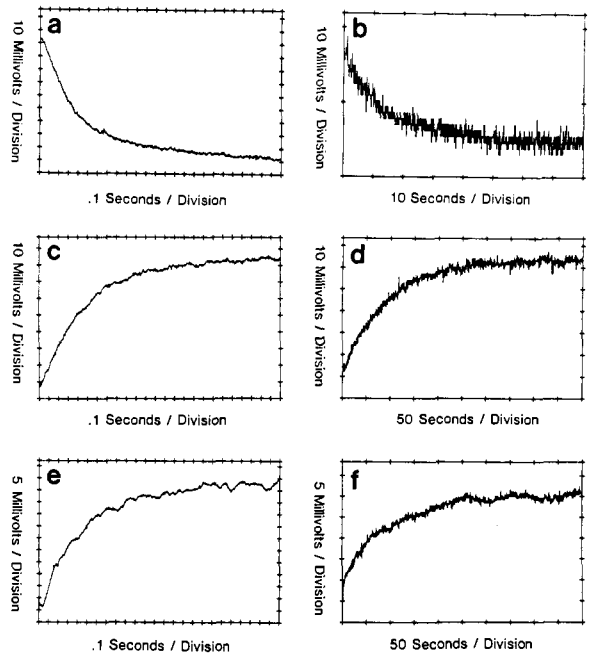

FIGURE 2: Stopped-flow measurements of refolding following a 2.0–0.4 M Gdn·HCl concentration jump at 20 °C. Folding monitored by fluorescence in the (a) τ_2 time range and (b) $\tau_1{}^b$ time range. Refolding monitored by absorbance changes at 287 nm in the (c) τ_2 time range and (d) $\tau_1{}^a$ time range. Refolding monitored by absorbance changes at 695 nm in the (e) τ_2 time range and (f) $\tau_1{}^a$ time range. All solutions contain 0.1 M sodium phosphate, pH 7.2.

[see Greenwood & Palmer (1965), Brandt et al. (1966), and Davis et al. (1974)]. For stopped-flow measurements of refolding using the 695-nm absorbance band a 500-nm cutoff filter was used in series with the monochromator. The final protein concentration for these experiments ranged from 3.4 × 10^{-5} to 10^{-4} M/L which is 7–20-fold higher than the concentrations used for other experiments.

Other methods and materials are as described previously (Nall & Landers, 1981).

Results

Temperature-Jump Measurements of Unfolding Kinetics. In Figure 1 oscilloscope traces are shown for the kinetic phases in unfolding that fall in the temperature-jump time range. Three different structural probes have been used to monitor folding: absorbance changes in the visible (Figure 1a,b), absorbance changes in the ultraviolet (Figure 1c,d), and changes in fluorescence (Figure 1e,f). The fastest kinetic phase is in the millisecond time range (τ_3) and is positively detected only by visible absorbance changes, being close to the level of detectability (if present at all) when monitored by the remaining two structural probes. The ability to detect phase τ_3 by visible absorbance changes but not by the other two structural probes holds for temperature-jump measurements throughout the Gdn·HCl-induced transition zone.

A slower kinetic phase in the seconds time range (τ_2) is easily detected by all three structural probes (Figure 1b,d,f). The time constants observed for this kinetic phase are essentially the same for the different structural probes although the accuracy and reproducibility of temperature-jump measurements in the seconds time range preclude discrimination between time constants which differ by less than 10–20%.

A relative amplitude α_3 can be calculated for τ_3 from

$$\alpha_3 = \Delta A_3/(\Delta A_2 + \Delta A_3)$$

where ΔA_3 and ΔA_2 are the total changes in absorbance (or fluorescence) associated with kinetic phases τ_3 or τ_2, respectively. At λ 418 nm, $\alpha_3 = 0.16$ while $\alpha_3 < 0.04$ at λ 287 nm and $\alpha_3 < 0.08$ for fluorescence. Therefore, α_3 is positively detected only by absorbance changes at λ 418 nm and, if present when monitored by fluorescence or absorbance in the ultraviolet, has a lower relative amplitude.

Stopped-Flow Measurements of Refolding. In Figure 2a–d kinetic measurements of refolding monitored by two different methods are compared. The initial conditions of the experiment are such that the protein is at the upper edge of the unfolding transition zone in a completely unfolded state. Refolding is induced by a 6-fold dilution of the Gdn·HCl by stopped-flow mixing such that the final equilibrium state is at the lower edge of the transition zone where the protein is fully folded. The fastest phase detected in the stopped-flow experiments is identified with the slower of the two kinetic phases detected by temperature-jump experiments (τ_2) since the time constants for this reaction are the same for both temperature-jump and stopped-flow experiments when the final conditions of the two experiments are the same (Nall & Landers, 1981). As can be seen, the fast phase in stopped-flow refolding has about the same time constant when monitored either by absorbance in the ultraviolet (Figure 2c) or by fluorescence (Figure 2a).

On the other hand the terminal phases in refolding fall into distinctly different time ranges when monitored by different structural probes. By fluorescence (Figure 2b), folding is essentially complete after about 30 s while ultraviolet absorbance changes (Figure 2d) persist for about 200 s. These terminal phases in folding have been designated $\tau_1{}^a$ (for absorbance) and $\tau_1{}^b$ (for fluorescence).

Refolding Monitored by Probes of the Native Conformation. For determination of which, if any, of the kinetic phases in refolding produce native protein, a functional assay has been used to monitor refolding. This method was developed by Ridge et al. (1981) and used to demonstrate that in the refolding of horse cytochrome *c* the native protein was produced in both fast and slow refolding reactions. The method is based on the fact that the rate of reduction of cytochrome *c* by ascorbate is about 10^3-fold less for the unfolded protein compared to that for the native protein (Ridge, 1978). Thus, under conditions where ascorbic acid concentrations are high enough that the bimolecular rate of reduction is faster than folding, heme reduction can be used to monitor the rate of formation of the native protein. In practice reduction is monitored by absorbance changes at 550 nm. This wavelength is close to being an isosbestic point for the Gdn·HCl-induced unfolding of oxidized iso-2 (Nall & Landers, 1981) but gives large absorbance changes on reduction of the heme.

Two kinetic phases are detected by ascorbic acid reducibility, a fast phase in the time range of kinetic phase τ_2 and a slow phase corresponding closely to that observed for refolding by absorbance, τ_1. However, control mixing experiments in which ascorbic acid was mixed with the native protein showed a slow reaction between ascorbic acid and the native protein which interfered with quantitation of phase τ_1. This may be due to residual protein which must undergo a slow isomerization reaction before being able to react with ascorbic acid. For horse cytochrome *c* an alkaline-induced isomerization of this type has been previously described (Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974). Thus we conclude that the product of phase τ_2 is reducible protein but must use other means for monitoring τ_1.

In Figure 2e,f results are presented for stopped-flow refolding monitored by changes in absorbance at 695 nm. This absorbance band is indicative of the presence of methionine as an axial heme ligand (Schechter & Saludjian, 1967) and is correlated with the native, ascorbic acid reducible form of cytochrome *c* [see Ridge et al. (1981)]. As can be seen in Figure 2e,f, both fast (τ_2) and slow (τ_1^a) phases are detected by absorbance changes at 695 nm. This confirms the fact that reducible protein is produced as the product of phase τ_2 but also indicates that kinetic phase τ_1^a produces native protein as a product.

Dependence of the Amplitudes and Time Constants on Guanidine Hydrochloride. In Figure 3 the relative amplitudes and time constants for folding–unfolding monitored by fluorescence are plotted as a function of the Gdn·HCl concentration in the final conditions of the kinetic experiments and are compared to previous results for folding–unfolding monitored by absorbance (Nall & Landers, 1981). For refolding experiments the protein was initially in a completely unfolded state in the presence of 2.0 M Gdn·HCl and then refolded by dilution of the Gdn·HCl by stopped-flow mixing to the indicated final concentration. For unfolding, the protein solution was initially in a completely folded state in the presence of 0.4 M Gdn·HCl and unfolded by the addition of Gdn·HCl to the indicated final concentration. As can be seen, the time constants are essentially the same for reactions monitored by either fluorescence or absorbance when the final conditions are near the midpoint of the transition zone, 1.15 M Gdn·HCl. Below the transition midpoint the fast phase (τ_2) has the same time constant for both fluorescence and absorbance, but the slow phase breaks up into two distinct kinetic phases, τ_1^a for absorbance and τ_1^b for fluorescence. The relative amplitude of the fast phase, α_2, is different for folding

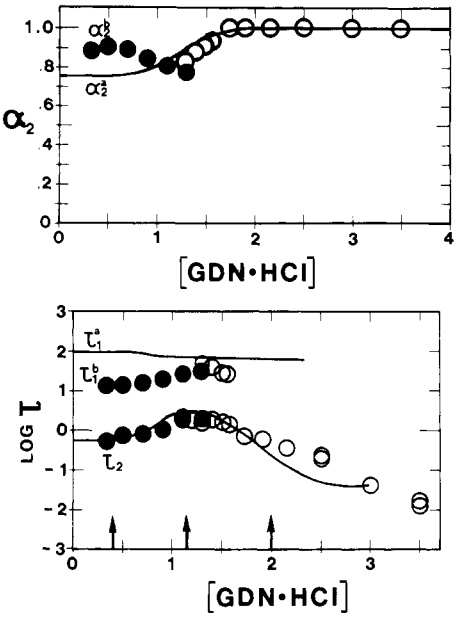

FIGURE 3: Relative amplitudes and time constants as a function of Gdn·HCl concentration for folding–unfolding kinetics of iso-2 monitored by fluorescence in the stopped flow. (Top panel) $\alpha_2 = \Delta F_2/(\Delta F_1 + \Delta F_2)$ where ΔF_i is the total change in fluorescence associated with kinetic phase *i*. (Bottom panel) Logarithm of the time constants (in seconds). Filled symbols (●) are for refolding experiments starting in 2.0 M Gdn·HCl and ending at the indicated Gdn·HCl concentration. Open symbols (○) are for unfolding experiments starting in 0.4 M Gdn·HCl and ending at the indicated concentration. Other conditions are 20 °C, 0.1 M sodium phosphate, pH 7.2. Arrows on the *x* axis indicate the beginning, midpoint, and end of the Gdn·HCl induced transition zone. Curves illustrating the kinetic behavior of α_2^a, τ_1^a, and τ_2 for a three-state model are shown for comparison (—). This model is successful in quantitatively describing folding–unfolding monitored by absorbance changes at 287 and 418 nm (Nall & Landers, 1981).

monitored by absorbance as compared to fluorescence below the transition midpoint. However, both α_2^a and α_2^b have inflections at about 1.0 M Gdn·HCl. At this same Gdn·HCl concentration τ_1^a and τ_1^b appear to merge into a single kinetic phase. Between 2.0 and 3.0 M Gdn·HCl there is an apparent difference between τ_2 monitored by fluorescence and absorbance. Experiments in which τ_2 for unfolding is compared for fluorescence and absorbance on the same samples show this difference to be insignificant (B. T. Nall, unpublished data).

Slow Refolding Monitored Spectrophotometrically. In Figure 4 difference spectra taken at 20-s intervals during refolding are presented. Although refolding is monitored simultaneously over the entire 230–800 nm range, only phase τ_1^a is observable since the faster phases (τ_2 and τ_3) are over within the mixing time of the experiment (5 s). Within the accuracy of the measurements the rate of refolding is the same in both the visible and ultraviolet spectral regions. This reconfirms and extends previous results (Nall & Landers, 1981) which showed that slow refolding monitored at selected wavelengths in the visible and ultraviolet occurred at the same rate. In addition (Figure 4, insert) the present results show that kinetic phase τ_1^a involves changes in absorbance of the 695-nm band, a spectral region believed to be indicative of the native conformation and heme ligands (Schechter & Saludjian, 1967).

Equilibrium and Kinetic Changes. In Table I relative amplitude and time constant data are presented for unfolding and refolding. For unfolding the conditions chosen start with the protein in the native state below the Gdn·HCl-induced tran-

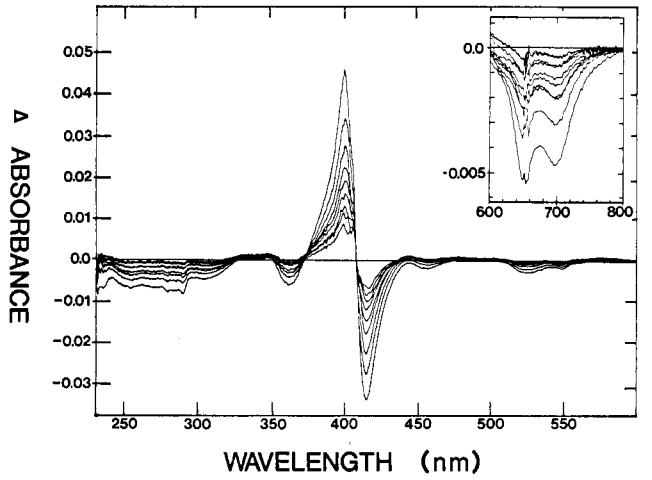

FIGURE 4: Slow refolding reaction of iso-2 cytochrome *c* monitored spectrophotometrically. Iso-2, initially unfolded in 2.0 M Gdn·HCl, was induced to refold by dilution to 0.33 M Gdn·HCl. Spectra, time averaged for 10 s, were taken at 20-s intervals. Difference spectra were obtained by subtracting the spectrum of the folded protein (taken 400 s after mixing) from spectra obtained while folding was in progress. The first nine difference spectra are shown. The final protein concentration was 15.7 μM (49.4 μM for the insert) with the initial unfolded protein concentration 6-fold higher. All solutions contained 0.1 M sodium phosphate buffer, pH 7.2. Temperature was 20 °C. The discontinuity at 650 nm is an instrumental artifact introduced by an optical filter.

Table 1: Comparison of Kinetic and Equilibrium Changes[a]

probe	τ_1 (s)	τ_2 (s)	α_2	$\Delta S_k/\Delta S_{eq}$[b]
refolding				
$\Delta F(350)$	11.5	0.75	0.83	0.38
$\Delta A(287)$	88.7	0.66	0.66	0.70
$\Delta A(695)$	81.1	1.1	0.77	1.30
unfolding				
$\Delta F(350)$		0.40	1	0.80
$\Delta A(287)$		0.33	1	0.44
$\Delta A(695)$		0.34	1	0.37

[a] Refolding is for a 2.0–0.5 M Gdn·HCl concentration jump while unfolding is for a 0.5–2.0 jump. Both are performed by mixing at a ratio of 1 to 5 in the stopped flow. Other conditions are temperature of 20 °C and 0.1 M sodium phosphate buffer, pH 7.2. [b] $\Delta S_k/\Delta S_{eq}$ is the fraction of the equilibrium signal change detected in the kinetic experiments. This includes the sum of the contributions from kinetic phase τ_1 and τ_2.

sition zone (at 0.5 M Gdn·HCl) and end at the upper edge of the transition (2.0 M Gdn·HCl). For refolding the protein is initially above the transition (2.0 M Gdn·HCl) and is refolded to a point just below the transition (0.5 M). Three spectral probes are compared, fluorescence at 350 nm, absorbance at 287 nm, and absorbance at 695 nm. In addition, static measurements of absorbance and fluorescence were made at the initial and final Gdn·HCl concentrations for the kinetic experiments allowing estimation of the fraction of the equilibrium signal change accounted for kinetically.

For refolding monitored by the three probes the fast phases have essentially the same rate. The slow phases monitored by the two absorbance probes also have the same rate. For fluorescence, however, the slow refolding reaction is complete in an 8-fold faster time range. Moreover, the relative amplitude for fluorescence refolding (α_2^b) is slightly larger than that found for the absorbance probes, and the fraction of the equilibrium fluorescence change accounted for is much less than one. This suggests the possibility of an additional fast phase in fluorescence-detected refolding outside the time range of the stopped-flow instrument (faster than 3 ms).

For unfolding, only a fast phase is reliably detected. Again, the rate of fast unfolding is the same for all three spectral probes. However, the fraction of the equilibrium change which is accounted for kinetically is well below one for the two absorbance probes but has increased to near one for fluorescence. This suggests the presence of additional fast phases in absorbance-detected unfolding which are outside the time range of the stopped-flow instrument.

Discussion

Kinetic Ratio Test. A powerful test for the presence of structural intermediates in folding is the kinetic ratio test (Labhardt & Baldwin, 1979). This test is simply the comparison of the time course of folding–unfolding reactions as monitored by different structural probes. When the ratio test is positive, that is, when different probes show different kinetic patterns, two conclusions may be drawn: (1) intermediate species are populated on the time scale of the experiment, and (2) the species have different properties when compared by the particular structural probes. Furthermore, to the extent that structure can be inferred from a given physical property, information is obtained about the nature of the intermediate species involved.

In applying this test, it must be kept in mind that all quantitative conclusions are limited by the sensitivity of the instrumentation. The present data show unequivocally that the relative amplitudes for the kinetic phases differ when monitored by distinct structural probes and thus that the conditions of the kinetic ratio test have been met. Improved instrumental sensitivity might allow the detection by fluorescence of phases presently detected only by absorbance (and vice versa). This possibility in no way compromises the fundamental experimental fact that different structural probes exhibit different kinetic patterns.

Folding Involves Fast Changes in Heme Environment. Application of the kinetic ratio test for the presence of folding intermediates shows that intermediates are present since the time course and relative amplitudes of folding reactions are different for different structural probes. The fastest observed folding–unfolding reaction for iso-2 is detected, at least preferentially, by heme absorbance changes. This demonstrates that species are populated on the time scale of τ_3 that have distinct heme absorbance properties. With the present data it is not possible to determine whether these species are kinetically populated species (transient intermediates) or species that are populated at equilibrium. However, τ_3 is detected for temperature jumps throughout the region of the Gdn·HCl transition zone (Nall & Landers, 1981). If one assumes that 5-deg temperature jumps satisfy the conditions for relaxation kinetics, then this suggests that these intermediate species may well be present at equilibrium.

Structural interpretations of the species must be consistent with the lack of significant ultraviolet absorbance or fluorescence changes. If ultraviolet absorbance changes are due to changes in the degree of exposure of aromatic groups to water and if fluorescence monitors an average heme to tryptophan distance (Tsong, 1974), then τ_3 reflects changes in heme environment which, to a first approximation, involve neither of these processes. Possibilities include exchange of heme ligands, localized configuration changes in the peptide backbone near the heme, or alterations in the polarity of the heme environment. Phase τ_3 might be due to the N → I or N → X_1 transitions described by Drew & Dickerson (1978) and Myer et al. (1980), respectively. These authors have postulated the existence of a less tightly folded form of the

native protein (I or X_1) based on spectrophotometric and circular dichroism studies of the equilibrium unfolding transition of horse cytochrome *c*.

It is surprising that phase τ_3 is not detected more readily by ultraviolet absorbance changes since the heme contributes the major fraction of the total absorbance in this region. Careful inspection of Figure 1c shows a slight curvature which, if real, may indicate the presence of this phase with an amplitude right at the level of detectability. Nevertheless, the relative amplitude (α_3) at 287 nm is well below that observed at 418 nm (at least 4-fold smaller).

Fluorescence Monitors the Formation of an Additional Intermediate. Applying similar criteria to stopped-flow refolding monitored by fluorescence demonstrates the existence of species with fluorescence close to that of the native protein but with intermediate ultraviolet absorbance properties. This is seen clearly from the fact that absorbance changes continue to occur after changes in fluorescence are complete (compare phases τ_1^a and τ_1^b). The terminal phase in folding monitored by absorbance changes at 695 nm, a probe indicative of the native conformation, is in the same time range as folding monitored by ultraviolet absorbance, suggesting that it is absorbance changes and not fluorescence changes which are correlated with the rate of formation of functional cytochrome *c* (Figures 2d,f and 4).

Care must be taken not to overinterpret these results. The fact that certain kinetic phases are not detected by particular spectral probes means only that the amplitudes associated with these phases are lower than the noise level. The kinetic phases, τ_1^a and τ_1^b, are of small amplitude and in a similar time range. This makes the resolution of a low amplitude τ_1^a phase monitored by fluorescence or a τ_1^b phase monitored by absorbance very difficult. Nevertheless, even if improved sensitivity allows detection of additional kinetic phases, the fundamental result remains unaltered: distinct kinetic patterns (i.e., different relative amplitudes) are observed for folding monitored by different physical and functional probes. This fact alone demonstrates the existence of spectrally distinct species during refolding.

It is interesting to compare the difference spectra of iso-2 for guanidine hydrochloride unfolding at equilibrium (Nall & Landers, 1981) with the kinetic difference spectra reported here (Figure 4). The relative magnitudes of the absorbance changes differ for the two experiments throughout the entire spectrum but are most noticeable in the ultraviolet region where the magnitude of the difference spectrum (relative to Soret changes) is larger for the equilibrium experiment than for the kinetic experiment. This could indicate aggregation and light scattering. If so, the scattering species must be formed transiently during refolding and then disrupted as folding proceeds since the equilibrium spectra have the expected absorbance ratios for the Soret maximum and the ultraviolet region. Furthermore, aggregation should give concentration-dependent kinetic properties. Since the same relative amplitudes and time constants are obtained for slow refolding monitored at 695 nm with 10^{-4} M/L iso-2 and at 287 nm with protein concentrations as low as 5×10^{-6} M/L iso-2 (Table I), there is no evidence of concentration dependence of the kinetic parameters. Spectral differences between the equilibrium and kinetic experiments are expected for at least two reasons: (1) Solvent effects will perturb the equilibrium but not the kinetic difference spectra. (2) The spectra are of two distinguishable types of species, the kinetic difference spectra being of partially folded species (with fluorescence close to that of the native protein) while the equilibrium difference spectra are of species which are unfolded by all optical criteria.

So what might be the properties of the intermediate species formed as a product of kinetic phase τ_1^b? Since these species have the fluorescence intensity of the native protein (1–5% of that of free tryptophan), a highly condensed state with a heme (quencher) to tryptophan (donor) distance similar to that of the native protein is indicated. Such an interpretation depends on the correlation Tsong has suggested (Tsong, 1974) for the fluorescence of cytochrome *c* and the overall protein conformation. Furthermore, the fact that these species show changes in ultraviolet absorbance on conversion to a functional native state indicates that the aromatic side chains in the intermediate species are, at least partially, exposed to solvent. These intermediates could be analogous to those observed in the folding of ribonuclease A where highly hydrogen bonded species with exposed aromatic groups have been detected (Schmid & Baldwin, 1979; Kim & Baldwin, 1980). For iso-2 there is no direct evidence for hydrogen-bonded structure, but the formation of such structure can be expected to require a highly condensed state of the protein.

It is of interest to compare the unimolecular refolding kinetics for iso-2 with the bimolecular fragment recombination–folding reactions for horse cytochrome *c* (Parr & Taniuchi, 1979). Like the present results, the kinetics of formation of productive (reducible) fragment complexes exhibit a unimolecular kinetic phase which is detected by absorbance but not by fluorescence. This unimolecular phase is not observed in the kinetics of formation of nonproductive (nonreducible) complexes.

Structural Traps and Slow Fold Reactions. The results of monitoring refolding by 695-nm absorbance and ascorbic acid reducibility show that native protein is formed in both fast and slow refolding reactions even when the initial state of the protein is above the unfolding transition zone. This is consistent with the mechanism

$$U_1 \overset{\text{slow}}{\rightleftharpoons} U_2 \overset{\text{fast}}{\rightleftharpoons} N$$

where both the fast and slow refolding reactions yield native (reducible) protein. Ridge et al. (1981) have reported similar results for horse cytochrome *c*. These authors also report multiple kinetic phases in the time range of phase τ_2. For iso-2 there is no clear evidence of complexity, but it is likely that our instrumentation is considerably less sensitive than that used by Ridge et al., so the existence of multiple fast refolding forms of iso-2 is not ruled out.

The nonnative protein remaining after the fast phase (τ_2) is complete must have differed initially from the fast refolding material in chain configuration rather than in organized intramolecular structure since both the fast and slow folding species of unfolded cytochrome *c* (at least those from horse) have hydrodynamic properties close to those of random-coil polypeptides (Ikai et al., 1973; Tsong, 1974). Possibilities include different cis or trans isomeric states about proline residues (Brandts et al., 1975) or differences in heme ligation (Henkins & Turner, 1979). Configurationally slow steps of this type may serve as structural traps allowing the transient accumulation of high concentrations of structured folding intermediates of the type detected as the product of kinetic phase τ_1^b.

So what can be learned from the characterization of the type of structure formed in slow refolding intermediates? What is the information content of these structured forms regarding the problem of furthering our understanding of the process of protein folding? There are two extreme points of view: (1)

these structured intermediates are aberrant species formed only when configurationally slow steps block the fast path to the native state, and (2) these same structured intermediates are on the direct pathway of folding in both the fast and slow folding reactions and are merely isolated in time by configurationally slow steps. At present there is little experimental evidence supporting either proposal except the observation that kinetic events attributed to configurationally slow steps are catalyzed by intramolecular structure (Kim & Baldwin, 1981). This tends to support the second viewpoint. If the latter proposal holds true, then the experimentally accessible study of structure formed in slow folding intermediates will lead directly to an understanding of the folding process for both fast and slow refolding species.

Additional Fast Phases? Comparison of the total kinetic changes to equilibrium changes (Table I, column 5) is plagued by two problems. First, the measurements themselves are intrinsically inaccurate since one must either measure the equilibrium changes on kinetic equipment designed for rapid response rather than long-term stability or make the measurements on a standard spectrophotometer against which the kinetic equipment has been calibrated. Both procedures can introduce substantial systematic errors. Second, the dependence of the measured optical properties for the native and unfolded protein on guanidine hydrochloride concentration within the transition zone can only be estimated by extrapolation. Nevertheless, for refolding it seems clear that only part of the equilibrium fluorescence change can be accounted for. A faster fluorescence change must be occurring within the dead time of the stopped-flow instrument (3–5 ms). Since the equilibrium change at 695 nm is adequately accounted for kinetically, the very fast fluorescence changes must lead to species lacking the 695-nm absorbance of the native protein.

Correspondingly, for unfolding the absorbance changes at 695 and 287 nm fall far short of the equilibrium values suggesting undetected, fast unfolding reactions which give rise to substantial absorbance changes.

Conclusions

At least two structured intermediates in the folding–unfolding reactions of yeast iso-2 cytochrome *c* have been shown to exist. One of these intermediates is detected by visible absorbance differences attributable to changes in heme environment or ligation. Apparently these changes occur in the absence of significant alterations in the polarity of the environment of aromatic groups or in the average heme to tryptophan distance since there are little, if any, detectable changes in either ultraviolet absorbance or fluorescence. At present it is not known whether this intermediate is present at equilibrium within the unfolding transition zone or whether it is formed only transiently during the folding process. If a transient intermediate, it is formed on a time scale of about 1 ms throughout the unfolding transition zone (Nall & Landers, 1981). A second intermediate is formed in a slow refolding reaction with a time constant of about 10 s. Since the formation of this species can be detected easily by fluorescence but not by absorbance, it is suggested that this is a highly condensed form of the protein with at least partially exposed aromatic groups.

The use of probes of the native conformation, ascorbic acid reducibility and 695-nm absorbance, to monitor the rate of formation of active sites indicates that functional protein is formed in both fast and slow refolding reactions. The time course of appearance of functional protein is apparently the same as for folding monitored by ultraviolet or Soret absorbance, suggesting that, in contrast to fluorescence, slow folding monitored by absorbance reflects the formation of native protein.

It is proposed that configurationally slow steps in folding such as proline isomerization or heme ligand exchange may serve as structural traps for the same intermediate species which refold on a faster time scale in the fast phases of folding. This proposal needs to be tested. If true, the experimentally accessible study of structured intermediates in slow refolding reactions will provide information about the process of folding in fast folding reactions as well.

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