Guanidine Hydrochloride Induced Unfolding of Yeast Iso-2 Cytochrome *c*†

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ABSTRACT: The properties of the guanidine hydrochloride induced unfolding transition of iso-2 cytochrome *c* (iso-2) from *Saccharomyces cerevisiae* have been investigated by using kinetic and equilibrium techniques and have been compared with previously published studies of horse cytochrome *c*, which differs from iso-2 by 46% in amino acid sequence. Measurements of absorbance in the ultraviolet and visible spectral regions as a function of guanidine hydrochloride concentration give superimposable equilibrium transition curves with a midpoint of 1.15 M at pH 7.2 and 20 °C. A two-state analysis of the equilibrium data gives a Gibbs free energy of unfolding of 3.1 kcal/mol at 20 °C in the absence of denaturant. This agrees well with the predicted difference in stability between *S. cerevisiae* iso-2 and horse cytochrome *c* estimated from the free energies of transfer of buried hydrophobic groups. Three kinetic phases associated with folding can be detected throughout most of the transition zone. Two of the phases are detected by stopped-flow mixing experiments. The third phase is over within the mixing time of the flow experiments but is detectable by temperature jumps. At 20 °C, pH 7.2, the slowest phase (τ_1) is in the 20–100-s time range, the middle phase (τ_2) is in the 0.1–3-s range, and the fastest phase (τ_3) is on the order of 1 ms. For the reactions observed in the stopped flow (τ_1 and τ_2), a simplified three-state mechanism can be used to predict quantitatively the relative amplitudes of the phases and the equilibrium unfolding curve from the observed time constant data. Previously this same mechanism has been successful in describing the folding reactions of horse cytochrome *c* [Hagerman, P. J. (1977) *Biopolymers 16*, 731]. We suggest that the qualitative features of protein folding reactions may be conserved among homologous proteins.

While it is known that the amino acid sequence of a protein contains the necessary information for the formation of a unique tertiary structure, how this information is encoded is not known. Two general approaches to this problem are of interest: (1) a comparison of the properties of the folding reactions for proteins with drastically different tertiary structures and (2) a comparison of homologous proteins with the same tertiary structure. The first approach suffers from the fact that for nonhomologous proteins it is very difficult to attribute any differences in folding to particular amino acid residues since the proteins differ in both sequence and tertiary structure. The second approach may allow one to attribute differences in folding to differences in sequence, but will it lead to an understanding of the folding process? In comparing homologous proteins, important properties of folding reactions are likely to be conserved along with the tertiary structure.

A related approach is to investigate the folding of altered proteins from organisms with structural mutations in the protein of interest. Such proteins are more likely to be defective in folding and thus provide stronger clues for discovering the rules which govern the folding process. This approach requires that the protein under study be the product of a gene and an organism with well-understood and easily manipulated genetic properties. Of low molecular weight monomeric proteins, the cytochrome *c*'s are the largest class of homologous proteins with known amino acid sequences. Among the cytochrome *c*'s, those from *Saccharomyces cerevisiae* have the most thoroughly developed genetics (Sherman & Stewart, 1978). For these reasons, we have undertaken a detailed study of the equilibrium and kinetic properties of the folding of iso-2 cytochrome *c* and mutants of iso-2[1] from *S. cerevisiae*. We report here the folding behavior of the wild-type protein.

Yeast contain two varieties of cytochrome *c*, iso-1, which accounts for 95% of the cytochrome *c*, and iso-2, which makes up the remaining 5%. These two proteins are believed to play identical physiological roles (Mattoon & Sherman, 1966) but are surprisingly divergent in amino acid sequence (17 amino acid differences plus four extra residues on the amino terminal end of iso-2; Montgomery et al., 1980). The major species, iso-1, contains a free sulfhydryl group which promotes covalent dimer formation in vitro. Iso-2 contains no free sulfhydryls and is, therefore, more suitable for folding studies of monomeric proteins. Although the X-ray crystallographic determination of the tertiary structure for the yeast proteins remains to be performed, structures have been determined for cytochrome *c*'s from a variety of eukaryotic and bacterial sources. The striking similarities between the cytochrome *c*'s from highly divergent organisms (Almassy & Dickerson, 1978) suggest that the yeast cytochrome *c*'s are likely to have tertiary structures very similar to that of cytochrome *c* from tuna.

While some equilbrium data are presented in what follows, we focus mainly on the kinetic properties of protein folding reactions since kinetic methods have been uniquely successful in demonstrating unambiguously the presence of a multitude of species during unfolding or refolding (Baldwin, 1975). Our approach is the same as that of Ikai & Tanford (1973) and Hagerman & Baldwin (1976). Stopped-flow mixing is used to create rapid guanidine hydrochloride (Gdn·HCl)[1] concentration changes which start either above or below the unfolding transition zone and which end within the transition zone. The time constants and relative amplitudes associated with the various kinetic phases of folding or unfolding are measured and then used along with the equilibrium unfolding curve to test whether or not particular kinetic mechanisms are compatible with the data. Similar studies have been performed on cytochrome *c* from horse (Ikai et al., 1973; Tsong, 1976), and a comparison is made between our results and the results for the protein from horse.

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

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Materials and Methods

The yeast strain used, B4926, was obtained from Dr. Fred Sherman of the University of Rochester. It contains a deletion of the structural gene for iso-1 cytochrome *c* and a mutation which causes 30-fold overproduction of iso-2 (Downie et al., 1977). The complete genotype is *MATα CYC7-H1 cyc1-1 trp1-1*.

The growth medium contained, per L, 10 g of Ardamine Z yeast extract (Yeast Products, Inc.), 10 g of dextrose, 34 mg of penicillin G, 100 mg of streptomycin, and 0.5 mL of antifoam B concentrate (Sigma). The inoculum (2–3 L) was a fresh culture, having been in stationary phase for no longer than 6 h. Yeast were grown in 180-L batches in a New Brunswick fermentor thermostated at 30 °C. Aeration was at a rate of 220 L/min, with agitation set at 300 rpm. The foam breaker was set at its maximum rate of 2400 rpm. After inoculation, growth was allowed to proceed until the cells were 3–6 h into stationary phase (about 24 h total), after which the yeast were harvested by centrifugation. The yield was 2–3 kg of wet cells. The yield can be doubled by the addition of 20 g of Bactopeptone (Difco) per L of media described above, but this greatly increases the expense of the media. The cells were either used immediately in a protein preparation or frozen and used within 2 weeks.

Protein Purification. Iso-2 cytochrome *c* was purified by the method of Sherman et al. (1968). The final step of this procedure involves elution of the protein from a cation-exchange column (Bio-Rex 70, 200–400 mesh) by a 0–0.8 M sodium chloride gradient. At this point, it was convenient to concentrate the protein before storage by (1) diluting the protein solution 10-fold by the addition of deionized water, (2) adding 30 g of Bio-Rex 70 (100–200 mesh) and stirring slowly for 20 min, (3) allowing the resin to settle out, and (4) decanting off the clear buffer. If the supernatant was still pink after the resin had settled out, steps 2 and 3 were repeated. The resin to which cytochrome *c* was bound was poured into a column, and the protein eluted in concentrated form with 0.8 M NaCl–0.1 M sodium phosphate buffer, pH 7.2. The resulting protein solution was about 10^{-3} M in cytochrome *c*. It was dialyzed against three changes of a 100-fold volume excess of 0.1 M sodium phosphate buffer, pH 7.2, and stored at −5 °C until use. Purity was checked by polyacrylamide gel electrophoresis (Laemmli, 1970) which indicated a single band of protein with a molecular weight of about 12 500 g/mol.

Equilibrium Unfolding by Guanidine Hydrochloride. Guanidine hydrochloride (Heico, Inc.) was used to prepare 6–7 M stock solutions in 0.1 M sodium phosphate buffer, pH 7.2. Small amounts of ultraviolet absorbing material were removed from the stock solutions by adding about 0.2 mg of activated charcoal per mL of stock solution and stirring for 1 h. The activated charcoal (Sigma) was removed by filtration through 0.65-μm membrane filters (Millipore Type DA). The concentration of the stock solution was determined by density (Sober, 1970) and used to prepare sample solutions of varying guanidine hydrochloride concentration and constant iso-2 concentration in 2-mL volumetric tubes.

Protein stocks were thawed, and the concentration was determined by absorbance measurements at 410 nm. The concentration was estimated by using the molar extinction coefficient of horse cytochrome *c* at 410 nm, $\epsilon = 106.1 \times 10^3$ L/(mol·cm) (Margoliash & Frohwirt, 1959). Protein was oxidized with a small molar excess of ferricyanide and passed over a Sephadex G-25 column to remove the oxidant. Sample solutions containing a constant concentration of iso-2 and varying amounts of guanidine hydrochloride were prepared in two ways. First, the protein stock aliquot was added to the appropriate volume of guanidine stock and gently mixed before 0.1 M sodium phosphate buffer was added to make up the final volume. In this manner, the protein was briefly exposed to a high enough concentration of guanidine hydrochloride to cause it to unfold. Second, the protein stock was added to the buffer and mixed, and finally the Gdn·HCl stock was added so that the protein was never exposed to high Gdn·HCl concentrations. Solutions prepared in either order gave the same transition curve, indicating that the system was at equilibrium. Final protein concentration was $(2–3) \times 10^{-6}$ mol/L.

The sample temperature was maintained at 20 ± 0.1 °C by using a circulating water bath connected to a thermojacketed sample cuvette. The temperature was monitored with a thermistor thermometer (Digitec Model 2780A). Complete spectra in the range 200–800 nm were obtained on a Hewlett-Packard 8450A UV–visible spectrophotometer, transferred to an Apple II Plus microcomputer, and stored on a magnetic disk.

Kinetic Measurements. Stopped-flow measurements were made by using a Durrum D-110 flow system in combination with a lamp housing, grating monochromator and quartz focusing optics (Oriel Corp.). The photomultiplier tube (Model 9558QB) and photomultiplier housing were from EMI-Gencom. A variable high-voltage power supply from Power Designs (Model 2K2D) was the high voltage source for the photomultiplier tube. Tungsten iodide lamps (Oriel Corp., 12 V DC, 100 W) were used as light sources. The lamp power supply was a Hewlett-Packard (Model 6267B) DC power supply operating in the constant current mode. Monochromator slit settings were adjusted to give a 20-nm band pass centered about the wavelength of interest (usually 287 or 418 nm). Stray light filters were unnecessary at the protein concentrations used. The effective light path was determined by taking absorbance measurements in the stopped-flow and the UV–visible spectrophotometer on a series of solutions spanning a wide range in protein concentration. This was done at both 287 and 418 nm, and the resulting calibration curves were used to correct absorbance changes observed in the stopped flow. Gas-tight syringes (Hamilton) were adapted to the Durrum flow system in order to allow different mixing ratios for concentration jump experiments. For the experiments reported here, the mixing ratio was 1:5. In the Durrum stopped-flow system, the drive syringes are mounted one above the other. When solutions of different densities were to be mixed, the syringe containing the more dense solution was mounted on the bottom to prevent premixing which gives rise to flow artifacts (Ikai, 1971). The drive syringes and the observation chamber were thermostated at 20 ± 0.1 °C by separate circulating water baths. The temperature of the drive syringes was monitored continuously with a thermistor thermometer, and the temperature of the observation chamber was adjusted to eliminate the thermal schlieren artifact observable in the seconds time range when there is a difference in temperature between the solutions entering the observation chamber and the observation chamber itself (Gibson, 1964). In this way, it is possible to compensate for the small change in temperature on dilution of concentrated guanidine hydrochloride solutions (Ikai, 1971).

The output of the photomultiplier was connected to an RC filtering and signal balancing network which allowed offset of the steady-state photomultiplier output (adjusted to 2.0 V with the photomultiplier power supply). The filtered and balanced signal was stored in a transient recorder (Biomation, Model 805) and monitored on an oscilloscope (Tektronix,

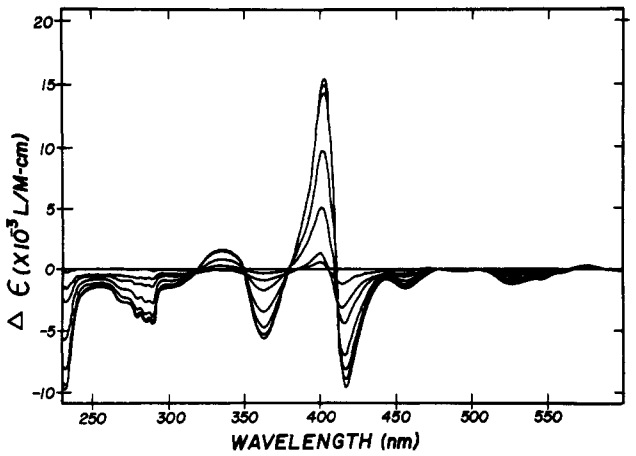

FIGURE 1: Difference spectra illustrating the Gdn·HCl-induced unfolding transition for iso-2 cytochrome *c*. All solutions contain 0.1 M sodium phosphate, pH 7.2, and are at 20 °C. The reference solution contains 0.5 M Gdn·HCl while the sample solutions in order of increasing spectral differences contain 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, and 2.1 M Gdn·HCl.

Model T922) connected to the analogue output of the transient recorder. The digitized data were transferred to a microcomputer (Apple II Plus) and stored on a magnetic disk.

Temperature-jump experiments were performed on an Eigen–Demaeyer temperature-jump instrument (Messanlagen Studiengesellschaft, m.b.h.). Teflon-lined cells requiring about 1.5 mL of sample solution were used. Sample solutions contained 0.1 M sodium phosphate buffer, pH 7.2, and variable amounts of guanidine hydrochloride. Protein concentrations were in the range of 3×10^{-5} mol/L. The sample cell was thermostated at 15 °C by a circulating water bath so that a 5 °C temperture jump would give a final temperature of 20 ± 0.5 °C. In this way, the final conditions of temperature jumps and stopped-flow concentration jumps were the same. This allows a direct comparison of observed time constants. All solutions, whether for temperature-jump or stopped-flow experiments, were degassed for 1 h, prior to use, by gentle stirring in a desiccator under reduced pressure.

The slow phase in unfolding and refolding was monitored on the UV–visible spectrophotometer using manual mixing to initiate concentration jump experiments. Thermojacketed cuvettes were used to maintain the sample temperature at 20 ± 0.1 °C.

Data Analysis. The time constants and amplitudes of the kinetic phases observed in stopped-flow and temperature-jump experiments were determined by using a computer-based graphic method. The data were scaled and displayed graphically. Time constant and amplitude parameters for a trial function of one or two exponentials were entered, and the trial function was plotted on the computer display for visual comparison to the data. Parameters were altered and new trial functions plotted until a satisfactory fit was achieved. Under no conditions was a function containing more than two exponentials necessary for fitting the data.

Time constants and amplitudes for slow kinetic processes measured in the UV–visible spectrophotometer were determined by converting the digitized data (originally in the form of absorbance vs. time) to the form $\log_{10}(\Delta A)$ vs. time by using the integral microprocessor of the spectrophotometer. The data were plotted, and the time constant and amplitude were determined from the slope and intercept, respectively.

Results

Equilibrium Unfolding. In Figure 1, Gdn·HCl-induced difference spectra are presented for iso-2 cytochrome *c* at pH

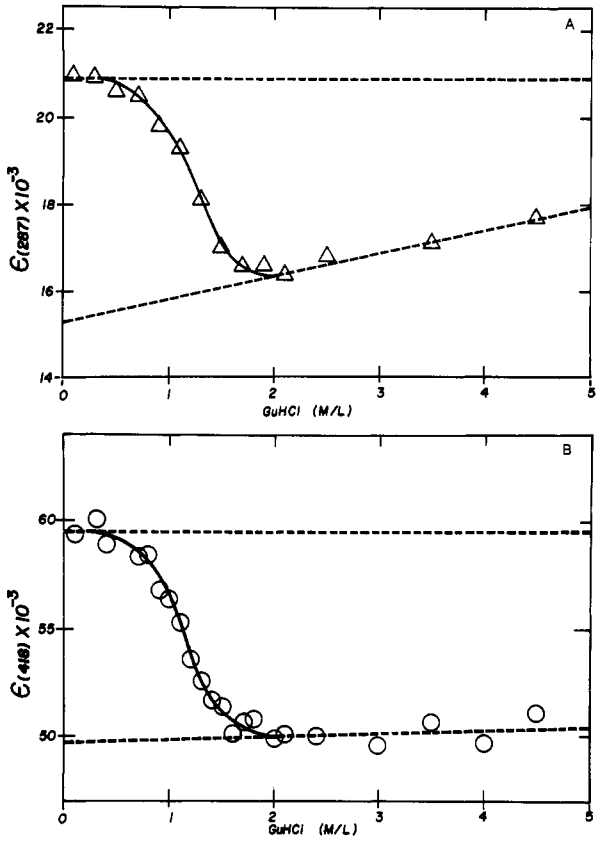

FIGURE 2: Molar extinction coefficient vs. the Gdn·HCl (GuHCl) concentration at (A) 287 (△) and (B) 418 nm (○). The assumed dependence of the extinction coefficient on Gdn·HCl concentration for the native and unfolded protein is shown (---). Conditions are described in Figure 1.

7.2, 20 °C. These spectra exhibit the same features observed for cytochrome *c* from other organisms in terms of the location of approximate isosbestic points, maxima and minima, and magnitudes of the total changes observed (Stellwagen, 1968; Ikai et al., 1973; Henkens & Turner, 1973; Kawaguchi & Noda, 1977). We have also monitored unfolding by using fluorescence (not shown) and have obtained results similar to those reported by Tsong (1976) for horse cytochrome *c*. In both cases, there is a change in fluorescence relative to that of free tryptophan from a few percent at low Gdn·HCl to about 40% at high Gdn·Hcl.

In Figure 2A, absorbance changes at 287 nm are plotted as a function of Gdn·HCl concentration. At this wavelength, it is expected that the changes should reflect alterations in the environments of the aromatic residues and the heme on exposure to solvent. In Figure 2B, the changes at 418 nm are given. This region of the spectrum is sensitive to changes in the environment of the heme alone. In both parts A and B of Figure 2, the assumed dependence of the extinction coefficient of the fully folded protein and of the fully unfolded protein on Gdn·HCl concentration is indicated. This allows the conversion of the optical parameters to the apparent fraction of folded protein as a function of Gdn·HCl concentration (Figure 3). The fact that the ultraviolet and visible optical parameters fall on the same curve is consistent with unfolding being a highly cooperative process in which the heme and the aromatic side chains are exposed to solvent simultaneously.

Kinetics of Refolding by Gdn·HCl Concentration Jumps. As can be seen from Figure 4A, the refolding of iso-2 cytochrome *c* induced by a Gdn·HCl concentration jump is a biphasic reaction. The fastest phase, τ_2, is of the order of

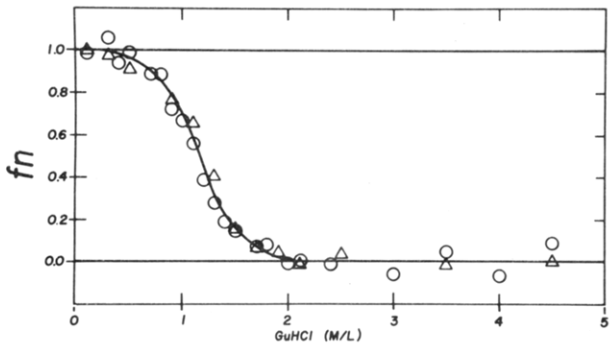

FIGURE 3: Fraction of folded iso-2 cytochrome *c* as a function of the Gdn·HCl (GuHCl) concentration. The fraction folded is calculated from the data in Figure 2 by using $f_n = (\epsilon - \epsilon_u)/(\epsilon_n - \epsilon_u)$, where ϵ, ϵ_u, and ϵ_n, respectively are the molar extinction coefficients of the protein solution and the extrapolated extinction coefficients for the unfolded and the native protein. Conditions are the same as in Figure 1. As in Figure 2, points are taken at 418 (○) and 287 nm (△). The smooth curve is from a three-state theoretical treatment of the time constant data (see Discussion).

seconds and accounts for 70% or more of the kinetically observable reactions in the time range of a stopped-flow experiment. The slower phase, τ_1, is of the order of 100 s for experiments ending below the transition zone but increases in rate by a factor of 3 or 4 for jumps ending at higher Gdn·HCl concentrations within the transition zone. The slower phase was monitored on the ultraviolet–visible spectrophotometer with manual mixing as well as by stopped flow since the base-line stability of the recording spectrophotometer gave more accurate data. The time constants and relative amplitudes of the kinetically observable phases in refolding are the same whether refolding is monitored by absorbance changes in the ultraviolet (287 nm) or visible (418 nm) region of the spectrum (Figures 6 and 7A).

Kinetics of Unfolding by Gdn·HCl Concentration Jumps. Unfolding of iso-2 by concentration jumps can be either biphasic or triphasic in the stopped-flow time range, depending upon the initial state of the protein. When freshly thawed samples of native protein are unfolded, three kinetic phases are observed. Two of the phases may be identified as the phases observed in refolding experiments. The correspondence of the two fastest unfolding phases to the phases observed on refolding is confirmed by performing unfolding experiments to the same final Gdn·HCl concentration as refolding experiments and comparing the time constants.[2] The third phase observed in unfolding is absent in refolding. This phase is very slow (300–1000 s) for experiments ending within the transition zone. The third phase may be completely eliminated by the following treatment: (1) addition of 0.4 M Gdn·HCl to the native protein, (2) brief thermal unfolding by exposure to 60–65 °C for 10 min, and (3) refolding by incubation at 20 °C for 10 min. Protein treated in this manner has the spectral properties of the native protein and exhibits biphasic unfolding kinetics when used within 10–20 min. The elimination of this kinetic phase proves that heat treatment removes nativelike species present before heat treatment. The nature of these species is unknown, but a possible explanation is suggested by experiments of Creighton (1980) in which polymeric forms of native horse cytochrome *c* were observed. These are eliminated by unfolding followed by refolding. Our results and analysis will be confined to heat-treated protein.

[2] Time constants, being functions of the macroscopic rate constants, are independent of the initial conditions of an experiment. The time constant associated with a particular kinetic phase must be the same for unfolding or refolding experiments as long as the experiments end in the same final state.

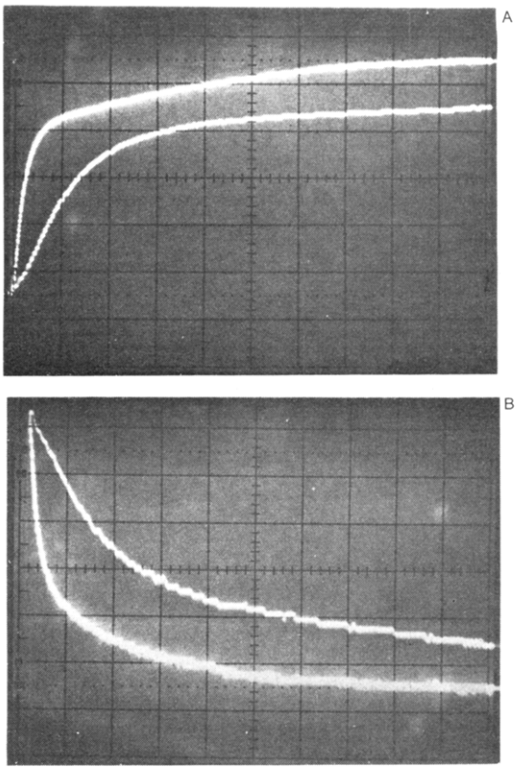

FIGURE 4: Light transmission changes as a function of time for (A) refolding following a 2.0–1.1 M jump in Gdn·HCl concentration and (B) unfolding following a 0.4–1.1 M jump in Gdn·HCl concentration. For (A), the vertical axis is 50 mV/division, while the horizontal axis is 10 s/division for the upper trace and 2 s/division for the lower trace. For (B), the vertical axis is 20 mV/division, the upper trace is 2 s/division, and the lower trace is 10 s/division. All solutions contain 0.1 M sodium phosphate, pH 7.2, at 20 °C.

As seen in Figure 4B, unfolding of heat-treated protein is a biphasic reaction, with most of the amplitude being associated with the faster of the two phases. The time constant for the fast phase (τ_2) is in the seconds time range for jumps ending within the transition zone but moves into the millisecond time range for jumps ending above the transition zone (Figure 6). Near the middle of the transition, the time constants obtained for unfolding and refolding experiments fall on the same curve and confirm a minimum in rate for τ_2 at the midpoint of the Gdn·HCl-induced transition. The time constant for the slow phase in unfolding, τ_1, is affected very little by Gdn·HCl concentration and remains in the 50-s time range. However, for unfolding ending above the midpoint of the transition zone, the slow phase loses amplitude and is no longer detectable above 1.8 M Gdn·HCl (Figure 7A). Similar time constant and amplitude behaviors are observed at both 287 and 418 nm (Figures 6 and 7A).

Temperature Jumps. Temperature jumps from 15–20 °C were performed at Gdn·HCl concentrations throughout the unfolding transition zone. The time range of the temperature-jump instrument (10^{-6}–10 s) allows overlap with the time range of the stopped flow (10^{-3} to $\sim 10^3$ s) and an extension of the search for kinetic events to reactions occurring 3 orders of magnitude faster. As can be seen from Figure 5, the fast phase, τ_2, detected by both unfolding and refolding in the stopped flow, is also detected by temperature jumps. The identification of this kinetic phase with τ_2 is confirmed by the fact that the time range and Gdn·HCl dependence of the time constant are the same as observed by stopped flow (Figure 6).

A third kinetic phase, τ_3, can be detected as well (Figure 5). This phase is associated with folding by the following

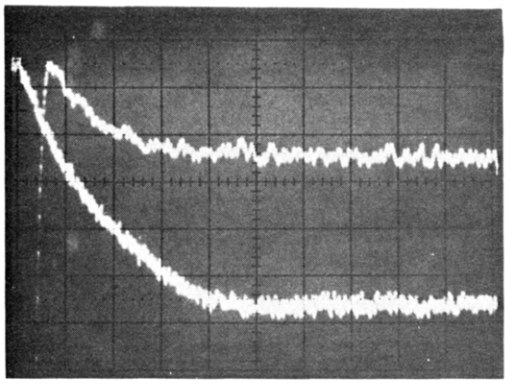

FIGURE 5: Temperature jump from 15 to 20 °C in 1.1 M Gdn·HCl and 0.1 M sodium phosphate, pH 7.2. The vertical axis is 4×10^{-3} absorbance unit per division, while the horizontal axis is 1 ms/division (upper trace) and 1 s/division (lower trace).

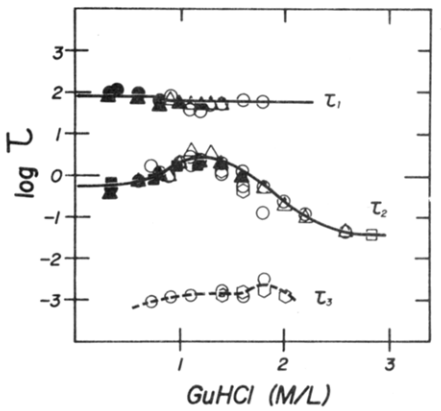

FIGURE 6: Logarithm of the time constants (in seconds) for folding and unfolding reactions of iso-2 cytochrome *c* as a function of Gdn·HCl (GuHCl) concentration. The wavelengths monitored are 418 (○), 287 (△), 359 (□), and 395 nm (⬡). Filled symbols indicate refolding experiments and open symbols unfolding experiments. Data points for τ_1 are by concentration jumps only, for τ_2 by temperature jumps and concentration jumps, and for τ_3 by temperature jumps only. The final conditions of the kinetic experiments are 0.1 M sodium phosphate, pH 7.2, at 20 °C and the indicated Gdn·HCl concentration. The assumed Gdn·HCl dependence of the time constants used along with a three-state mechanism to predict the relative amplitude behavior (Figure 7A) and equilibrium unfolding curve (Figure 3) is indicated (—). The dashed line through the data for τ_3 has no theoretical significance.

criteria: (1) it is present for temperature jumps starting or ending within the transition zone and (2) it is absent for jumps which both start and end either above or below the transition zone. The time constant is of the order of 10^{-3} s and shows little dependence on Gdn·HCl concentration (Figure 6). The relative amplitude for this phase, α_3, shows a slight dependence on Gdn·HCl concentration, dropping from about 50% of the observable kinetic change at either extreme of the transition zone to about 15% near the middle of the transition (Figure 7B).

Discussion

Unfolding Is Complete and Reversible. The Gdn·HCl-induced unfolding of iso-2 cytochrome *c* is a reversible process, as judged by the fact that the spectral properties of the native protein are regained on removal of the Gdn·HCl. Furthermore, the transition curve (Figure 3) is an equilibrium transition curve since the spectral properties of this protein depend only on the Gdn·HCl concentration and not on the previous history of the sample. The argument that we are monitoring an unfolding process is based on a comparison with cytochrome

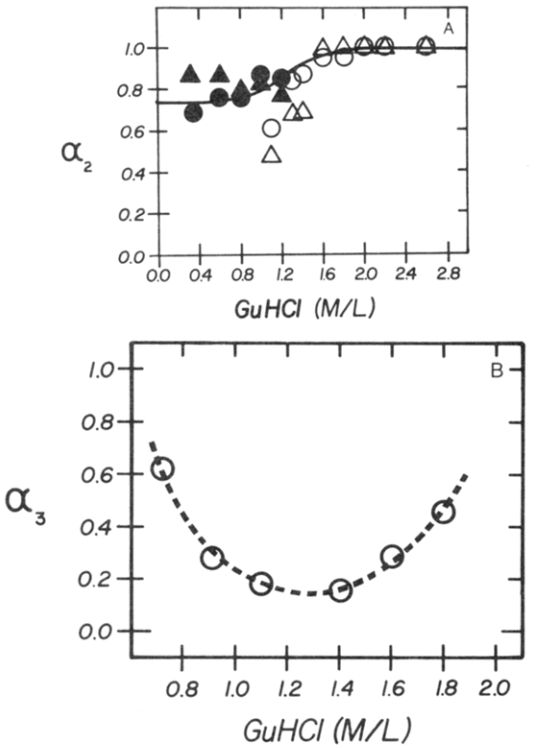

FIGURE 7: Relative amplitudes of kinetic phases observed by (A) Gdn·HCl (GuHCl) concentration jumps and (B) temperature jumps. For (A) $\alpha_2 = \Delta A_2^0/(\Delta A_1^0 + \Delta A_2^0)$, and for (B) $\alpha_3 = \Delta A_3^0/(\Delta A_2^0 + \Delta A_3^0)$, where ΔA_i^0 is the total change in absorbance associated with kinetic phase *i*. Open symbols indicate unfolding experiments and filled symbols indicate refolding experiments. Wavelengths monitored are 418 (○) and 287 nm (△). In (A), the relative amplitude behavior predicted by a three-state mechanism is shown (—). The dashed line in (B) has no theoretical significance.

c from horse for which a much wider range of properties has been investigated and interpreted in terms of an unfolding of the protein to a state approximating a random coil (Ikai et al., 1973; Stellwagen, 1968). Increases in tryptophan fluorescence have been shown to be correlated with changes in intrinsic viscosity for the unfolding of horse cytochrome *c* (Tsong, 1974). These fluorescence changes are the expected consequence of increases in the average distance between the fluoresent tryptophan residue (donor) and the heme (quencher). Measurements of the unfolding of iso-2 cytochrome *c* give the same relative fluorescence above 2.0 M Gdn·HCl as is obtained for horse cytochrome *c* above 3.5 M Gdn·HCl (Tsong, 1976). This indicates that the average heme–tryptophan distance is similar in the unfolded states of the two proteins. The product of the Gdn·HCl unfolding of horse cytochrome *c* has the intrinsic viscosity expected for the random coil state (Ikai et al., 1973), but the heme iron remains coordinated with two strong field ligands, presumably histidine side chains (Babul & Stellwagen, 1971; Tsong, 1975). Thus, a comparison of the spectral and fluorescence properties of iso-2 cytochrome *c* above 2.0 M Gdn·HCl and cytochrome *c* from horse above 3.5 M Gdn·HCl suggests a similar unfolded state for the yeast protein.

Is Unfolding a Two-State Process? The sensitivity of the visible spectrum to the environment and state of ligation of the heme has allowed the detection of several distinct states of horse cytochrome *c* (Schejter & George, 1964; Myer, 1968; Stellwagen, 1968; Kaminsky et al., 1973; Drew & Dickerson, 1978). Nevertheless, the major cooperative unfolding transition observed by differential scanning microcalorimetry appears to be a two-state process (Privalov & Khechinashvili, 1974). Creighton (1979) has proposed that these additional

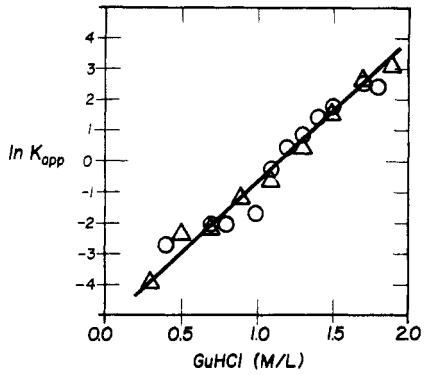

FIGURE 8: Natural logarithm of the apparent equilibrium constant for unfolding, $K_{app} = (1 - f_n)/f_n$, as a function of the Gdn·HCl (GuHCl) concentration. The data are from Figure 3. Symbols indicate the wavelength, 418 (○), or 287 nm (△). The line through the data has a slope of 4.67 mol^{-1} and intersects the 0 of the ln K_{app} axis at 1.14 M Gdn·HCl.

optical states are due to perturbations of the fully folded or unfolded protein by heme ligands rather than to additional conformational states. Careful inspection of the Gdn·HCl-induced difference spectra of yeast iso-2 (Figure 1) shows (1) poorly defined isosbestic points and (2) drift in the wavelength associated with the largest negative $\Delta\epsilon$ from about 410 to 418 nm. Neither of these effects would be expected if a simple two-state mechanism applies. This may be due to small contributions of other folding intermediates or more likely to solvent effects on the chromophores.

The Gibbs Free Energy for Unfolding Is Small. The Gibbs free energy of stabilization of iso-2 cytochrome *c* can be estimated by a two-state analysis of the data presented in Figure 3. A two-state treatment of equilibrium unfolding transitions for small globular proteins is justified on empirical grounds (Lapanje, 1978) and is not necessarily in conflict with the existence of multiple species in a kinetic experiment. We have used the method of Schellman (1978) for analyzing solvent denaturation processes to obtain the free energy of unfolding in the absence of denaturant. For proteins that exhibit a linear relationship between the free energy of unfolding and the molarity of Gdn·HCl this procedure is merely an extrapolation to 0 molarity. The method has been justified on thermodynamic grounds (Schellman, 1978) and has the advantage over the other procedures (Tanford, 1969) in that there are no assumptions regarding binding of denaturant to either the folded or the unfolded form of the protein. In Figure 8, the relationship between the natural logarithm of the apparent equilibrium constant for unfolding and Gdn·HCl molarity is shown to be linear. From Schellman (1978) we have

$$\Delta G^\circ = -RT\Delta b_{23}{}^0 C_m \quad (1)$$

where ΔG° is the Gibbs free energy of unfolding in the absence of denaturant and C_m is the denaturant concentration at the midpoint of the transition

$$\Delta b_{23} = [\partial (\ln K_{app})/\partial C_3]_{T,P} \quad (2)$$

where C_3 is the denaturant concentration in moles/liter and K_{app} = unfolded protein/native protein is the apparent equilibrium constant for unfolding. When these equations are used, a free energy for unfolding of +3.1 kcal/mol is obtained for iso-2 cytochrome *c* in the absence of denaturant at pH 7.2 and 20 °C. For horse cytochrome *c*, the two-state treatment of Gdn·HCl-induced transitions gives values of 7.27 (Knapp & Pace, 1974) and 7.38 kcal/mol (McLendon & Smith, 1978) near neutral pH at 25 °C.[3] Privalov & Khechinashvili (1974) report a value of 9 ± 0.6 kcal/mol from a microcalorimetric study at pH 4.8. So the free energy of stabilization of iso-2 cytochrome *c* is surprisingly small in comparison to that of horse.

Table I: Free-Energy Contributions to Stability

group	amino acid side chain	ΔG_{tr} (cal/mol)[a]
I	Tyr, Leu, Ile, Phe, Pro, Trp	2500
II	Val, Met, Lys (nonpolar part)	1500
III	Ala, Arg (nonpolar part)	600
IV	Glu, Asn, Gln, Ser, Thr, Cys	0
V	Asp, Glu, His, (charged part of Arg and Lys)	0

[a] The ΔG_{tr} are the estimated free-energy contributions (per mole) to the stability of the native structure for an amino acid side chain from each group. These values are from Brandts (1964) and Kawaguchi & Noda (1977).

Table II: Buried Hydrophobic Residues[a]

protein	no. of residues in group I	II	III
horse cytochrome *c*	23	2	1
iso-2 cytochrome *c*	20	4	3

[a] Buried residues are those at homologous positions to those classified as buried or partially buried in tuna cytochrome *c* (Dickerson & Timkovich, 1975). Heme has not been included.

The Free-Energy Difference between Iso-2 and Horse Cytochrome c Is Consistent with Difference in Sequence. An attempt can be made to attribute this difference in stability to differences in the primary structure of the two proteins along the lines of Tanford (1962). One of the most striking differences is the presence of ten amino acid residues at the amino terminus of iso-2 which are not present in cytochrome *c* from horse. As a first approximation, we will assume that this extra region is unstructured and does not add to or detract from the overall stability of iso-2. This assumption is supported by the fact that mutations near the amino terminus of the homologous iso-1 cytochrome *c* from yeast do not affect the function of that protein (Sherman & Stewart, 1978). Second, only those positions with buried or partially buried side chains will be compared. The decision as to whether or not a particular residue is buried is based on whether or not the residue in the analogous position in tuna cytochrome *c* is classified as being buried (Dickerson & Timkovich, 1975). Finally, the various amino acid residues are divided into classes depending on their hydrophobicity following Brandts (1964) and, in particular, Kawaguchi & Noda (1977). In this scheme, the amino acids are divided into five classes (Table I). It is postulated that only the three most hydrophobic groups make a significant contribution to the overall stability (Kawaguchi & Noda, 1977; Brandts, 1964). In Table II the number of buried or partially buried residues for cytochrome *c* from horse and iso-2 from yeast is compared group by group. An estimate of the average contribution to stability of a single residue from each group is given in Table I. When the contributions from hydrophobic groups for the two proteins are added up, the difference in the stability is estimated to be 3.3 kcal/mol at 25 °C. When the Gibbs free energy of unfolding of cytochrome *c* from horse

[3] These free-energy values were obtained by a linear extrapolation of the apparent free energy for unfolding to 0 M guanidine hydrochloride [the method suggested by Schellman (1978)]. Knapp & Pace (1974) treat the same data using the model of Tanford (1969) in addition to using the linear extrapolation model. Using Tanford's model, they obtain a much higher ΔG° for unfolding (12.7 kcal/mol).

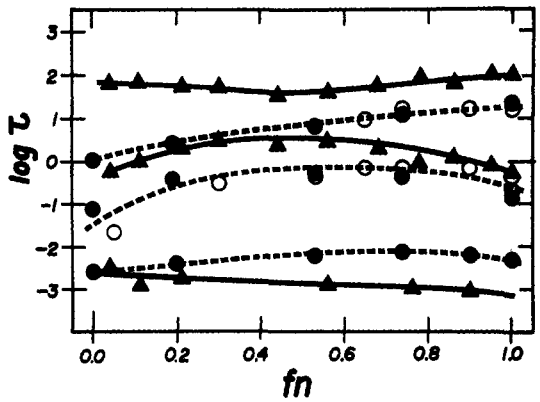

FIGURE 9: Dependence of the logarithm of the time constants on the fraction of folded protein present in the final conditions for iso-2 cytochrome *c* (–▲–) and horse cytochrome *c* (---●---). The horse cytochrome *c* data are from Tsong (1976) (●) and from Ikai et al. (1973) (○).

is taken to be 7.3 kcal/mol, the Gibbs free energy of unfolding of iso-2 is predicted to be 4.0 kcal/mol at 25 °C in the absence of denaturant. This may be compared with the experimental value of 3.1 kcal/mol derived from a two-state treatment of the equilibrium data obtained at 20 °C. Important factors have been ignored in this treatment, but the qualitative prediction is in good agreement with the experiment.

The Kinetic Properties Are Similar to Those of Other Small Globular Proteins. The kinetics of folding or unfolding of iso-2 cytochrome *c* are complex, as is the case for other proteins. In trying to understand a complex kinetic system, it is helpful to focus on the prominent features of the reactions. This requires the development of criteria for determining which features of folding reactions are, in fact, the prominent ones. A comparison of the kinetic properties of related proteins may be of help since the most important features of folding are expected to be the same for homologous proteins. On the other hand, to the extent that there are universal rules or mechanisms which guide the folding of all proteins, one expects to find similarity in the folding properties of unlike proteins as well. In Figure 9, the time constants for iso-2 cytochrome *c* from yeast are compared to those of cytochrome *c* from horse (Tsong, 1976; Ikai et al., 1973). For compensation for the difference in stability of the two proteins, the data are presented in the manner of Tsong (1976) as a function of the fraction of folded protein present in the final conditions of the experiment. While there are differences in the magnitudes of the time constants for the two proteins, the functional form is similar. The behavior of α_2 as a function of Gdn·HCl concentration for iso-2 is also similar to that of horse cytochrome *c* [see Ikai et al. (1973) and Hagerman (1977)]. On the other hand, the qualitative features of the kinetics are not unlike those reported by Hagerman & Baldwin (1976) for an analysis of the unfolding and refolding kinetics of the nonhomologous protein, ribonuclease A. Ribonuclease A exhibits three kinetic phases for both unfolding and refolding but a stronger dependence of the time constants on the final conditions.

The Relative Amplitude Behavior of the Two Slowest Phases Is Explained by a Three-State Mechanism. The similarities between the kinetic properties of the folding reactions of cytochrome *c* from horse and iso-2 from yeast suggest that a simplified three-state mechanism previously successful in describing the kinetic behavior of cytochrome *c* from horse (Hagerman, 1977) and ribonuclease A (Hagerman & Baldwin, 1976) might also apply to iso-2 cytochrome *c*. A complete analysis of the application of this mechanism to cytochrome *c* from horse has been given by Hagerman (1977) and will be given in outline form here. The mechanism is

$$U_1 \underset{k_{21},\text{slow}}{\overset{k_{12}}{\rightleftharpoons}} U_2 \underset{k_{32},\text{fast}}{\overset{k_{23}}{\rightleftharpoons}} N \qquad (3)$$

where U_1 and U_2 are kinetically distinguishable unfolded species with identical spectral properties and N is the native protein. The equilibrium between U_1 and U_2 is assumed to be independent of Gdn·HCl concentration. When $\tau_1/\tau_2 \gg 1$ and k_{23}, $k_{32} \gg k_{12}$, k_{21}, the time constants are given by

$$\frac{1}{\tau_1} \simeq k_{12} + \frac{k_{21}}{1 + K_{23}} \qquad (4)$$

$$\frac{1}{\tau_2} \simeq k_{23} + k_{32} \qquad (5)$$

where K_{23} is the equilibrium constant for the U_2 to N reaction. For refolding experiments starting above the transition zone and unfolding experiments starting below the transition zone, the fraction of fast refolding (or unfolding) material, α_2, is given by

$$\alpha_2 = \frac{1}{1 + \dfrac{|1 - \tau_1(k_{12} + k_{21})|}{|1 - \tau_2(k_{12} + k_{21})|}} \qquad (6)$$

For refolding under the conditions given for eq 4 and 5, the relative amplitude of the fast reaction, α_2, is given by

$$\alpha_2 = U_2^0\left(1 + \frac{K_{21}}{1 + K_{23}}\right) \qquad (7)$$

where U_2^0 is the fraction of fast refolding protein present in the initial conditions of the experiment and K_{21} is the equilibrium constant for the U_2 to U_1 reaction.

From eq 6, once k_{12} and k_{21} are determined, α_2 can be calculated from time constant data throughout the transition zone (where k_{12} and k_{21} are assumed to be independent of denaturant). Since all of the Gdn·HCl dependence of the transition is attributed to K_{23}, the value of K_{23} must be very large for refolding experiments ending at Gdn·HCl concentrations where the protein is fully folded. Under these conditions, eq 4 and 7 reduce to

$$\frac{1}{\tau_1} \simeq k_{12} \qquad \alpha_2 \simeq U_2^0$$

Since $K_{12} = U_2^0/U_1^0$, all of the parameters are determined. The lines drawn through the time constant data for τ_1 and τ_2 in Figure 6 have been used to generate the theoretical relative amplitude behavior shown by the smooth line in Figure 7A. Extending this approach, Hagerman & Baldwin (1976) have developed equations which allow the prediction of the equilibrium unfolding curve from knowledge of k_{12}, k_{21}, τ_1, and τ_2. These equations have been used to predict the theoretical transition curve given in Figure 3. While the fit seems good in both cases, the predicted curves are very sensitive to the parameter τ_1. A small variation in the values chosen for τ_1, even well within experimental errors, can cause serious disagreement between the experimental data and the predicted equilibrium unfolding curve and relative amplitudes.

For unfolding experiments ending near the midpoint of the Gdn·HCl unfolding transition, there is a tendency for α_2 to fall below the predicted curve (Figure 7A). Since the total changes in absorbance are smallest for kinetic experiments ending in this part of the transition, the relative amplitudes in this range are much more susceptible to systematic errors.

It is our view that this discrepancy between experiment and theory is of marginal significance. Assuming that the discrepancy is real, then we have a situation where α_2 (refolding) $\neq \alpha_2$ (unfolding). Within the framework of the three-state mechanism, this implies a change in the U_1 to U_2 equilibrium on going from the initial conditions of the experiment to the final conditions (Hagerman & Baldwin, 1976). It is reasonable that the change in Gdn·HCl concentration in these experiments (0.4 to near 1.0 M) might well affect the degree of any residual structure in unfolded species and thus shift the equilibrium between unfolded species to less structured forms. In comparing our results to those obtained by Ikai et al. (1973) for horse cytochrome *c*, it is interesting to note that α_2 (refolding) $\neq \alpha_2$ (unfolding) near the midpoint of the transition for that protein as well, with the deviations from the behavior predicted by the three-state model being similar to those observed for iso-2 [see Hagerman, (1977)].

A more serious problem with the application of this model is that τ_3 has been ignored. In principle, the presence of the third kinetic phase requires the existence of at least four species during the kinetic experiment, so the quantitative agreement between theory based on a three species mechanism and our experiments is somewhat surprising. It may be that agreement is accidental and that even the basic features of the three-state mechanism do not reflect the actual situation. A more likely possibility is that the fast reaction is a transient phase after which the three-state mechanism is a good approximation. The success of the three-state mechanism in describing the kinetic properties of such different proteins as lysozyme, ribonuclease A, and cytochrome *c* (Hagerman, 1977) tends to support the latter interpretation.

Nature of the Slow Refolding Reaction. Henkens & Turner (1979) proposed that the slow phase of refolding is due to heme–ligand interactions which lead to the formation of an incorrectly folded state. The slow disruption of the improperly folded protein and its ultimate conversion to the native state give rise to the slow refolding reaction. This suggestion is supported by the finding that iron-free porphyrin cytochrome *c* refolds in a single fast phase while ferricytochrome *c* refolds in a biphasic reaction involving a fast and a slow reaction. Another possibility consistent with monophasic refolding of porphyrin cytochrome *c* is direct involvement of a heme–ligand exchange reaction as a rate-limiting step in the slow refolding of ferricytochrome *c* [see Discussion in Ridge et al. (1981)].

A more general explanation has been offered by Brandts et al. (1975) who suggested that a slow cis $\rightleftarrows$ trans isomerization of proline residues about the imide bond might be the rate-limiting step in the slow refolding reactions of proteins. In the strict form of this model it is assumed that any proline residue in a nonnative isomerization state will completely block folding. However, recent theoretical (Levitt, 1981) and experimental (Jullien & Baldwin, 1981; Cook et al., 1979) work indicates that there are three classes of proline residues: (1) those which block folding until isomerization occurs, (2) those which slow down folding, and (3) those which have little effect on folding since they can be accommodated in the native structure almost equally well in either the cis or trans configuration.

For both horse (four prolines) and iso-2 (five prolines) cytochrome *c*'s, the observed relative amplitudes for complete refolding (α_2 = 0.7–0.8) are inconsistent with a strict formulation of the model of Brandts et al. (1975) but are consistent with the modified model in which only one or two of the prolines is capable of blocking folding. The involvement of prolines in slow folding is supported by the results of Ridge et al. (1981) who present strong evidence that horse cytochrome *c* obeys the mechanism we have used to analyze the iso-2 folding kinetics (eq 3). They find that the properties of the $U_1 \rightleftarrows U_2$ reaction are consistent with proline isomerization. These results tend to underscore the similarity of the slow reactions for horse and iso-2 cytochrome *c* but leave open the question of whether prolines, heme ligands, or other factors are involved in the slow steps in folding.

Conclusions

We have shown from the reversible unfolding transition of iso-2 cytochrome *c* from *Saccharomyces cerevisiae* that this protein is considerably less stable toward denaturation by Gdn·HCl than is the homologous cytochrome *c* from horse. The estimated differences in stability of the two proteins in the absence of denaturant are in reasonable agreement with theoretical expectations based on the numbers and types of buried hydrophobic groups.

The kinetic properties of the folding reactions of iso-2 are qualitatively the same as those of cytochrome *c* from horse (Ikai et al., 1973; Tsong, 1976). Two kinetic phases are observed in the time range of the stopped flow, and the amplitude behavior of these phases is quantitatively predicted by a three-state kinetic mechanism. The same kinetic mechanism is successful in predicting the equilibrium unfolding curve. A third kinetic phase has been detected by temperature jumps in a time range too fast for detection by stopped flow. Analogous fast phases outside the stopped-flow time range have been reported previously by Tsong (1976) for horse cytochrome *c*. Since cytochrome *c* from horse and iso-2 from yeast differ by 46% in amino acid sequence, but probably share the same tertiary structure, it seems reasonable to conclude that homologous proteins have homologous folding properties.

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References

Almassy, R. J., & Dickerson, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2674.

Babul, J., & Stellwagen, E. (1971) *Biopolymers 10*, 2359.

Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453.

Brandts, J. F. (1964) *J. Am. Chem. Soc. 86*, 4302.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157–6161.

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.

Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61.

Dickerson, R. E., & Timkovich, R. (1975) *Enzymes, 2nd Ed. 2*, 397.

Downie, J. A., Stewart, J. W., Brockman, N., Schweingruber, A. M., & Sherman, F. (1977) *J. Mol. Biol. 113*, 369.

Drew, H. R., & Dickerson, R. E. (1978) *J. Biol. Chem. 253*, 8420–8427.

Gibson, Q. H. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Gibson, Q. H., Eisenhardt, R. H., & Lonberg-Holm, K. K., Eds.) p 115, Academic Press, New York.

Hagerman, P. J. (1977) *Biopolymers 16*, 731.

Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462.

Henkens, R. W., & Turner, S. R. (1973) *Biochemistry 12*, 1618.
Henkens, R. W., & Turner, S. R. (1979) *J. Biol. Chem. 254*, 8110–8112.
Ikai, A. (1971) Ph.D. Thesis, Duke University, Durham, NC.
Ikai, A., & Tanford, C. (1973) *J. Mol. Biol. 73*, 145.
Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165.
Jullien, M., & Baldwin, R. L. (1981) *J. Mol. Biol. 145*, 265–280.
Kaminsky, L. S., Miller, V. J., & Davison, A. J. (1973) *Biochemistry 12*, 2215–2221.
Kawaguchi, H., & Noda, H. (1977) *J. Biochem. (Tokyo) 81*, 1307.
Knapp, J. A., & Pace, C. N. (1974) *Biochemistry 13*, 1289.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lapanje, S. (1978) in *Physicochemical Aspects of Protein Denaturation*, Wiley, New York.
Levitt, M. (1981) *J. Mol. Biol. 145*, 251–263.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570.
Mattoon, J. R., & Sherman, F. (1966) *J. Biol. Chem. 241*, 4330.
McLendon, G., and Smith, M. (1978) *J. Biol. Chem. 253*, 4004.
Montgomery, D. L., Leung, D. W., Smith, M., Shalit, P., Faye, G., & Hall, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 541.
Myer, Y. P. (1968) *Biochemistry 7*, 765–776.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665.
Ridge, J. A., Baldwin, R. L., & Labhardt, A. M. (1981) *Biochemistry 20*, 1622–1630.
Schejter, A., & George, P. (1964) *Biochemistry 3*, 1045.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Sherman, F., & Stewart, J. W. (1978) in *Biochemistry and Genetics of Yeast* (Bacila, M., Horecker, B. L., & Stoppani, A. O. M., Eds.) p 273, Academic Press, New York.
Sherman, F., Stewart, J. W., Parker, J. H., Inhaber, E., Shipman, N. A., Putterman, G. J., Gardisky, R. L., & Margoliash, E. (1968) *J. Biol. Chem. 243*, 5446.
Sober, H. A. (1970) in *Handbook of Biochemistry* (Sober, H. A., Ed.) 2nd ed., p J278, Chemical Rubber Co., Cleveland, Ohio.
Stellwagen, E. (1968) *Biochemistry 7*, 2893–2898.
Tanford, C. (1962) *J. Am. Chem. Soc. 84*, 4240.
Tanford, C. (1969) *Adv. Protein Chem. 24*, 1.
Tsong, T. Y. (1974) *J. Biol. Chem. 249*, 1988–1990.
Tsong, T. Y. (1975) *Biochemistry 14*, 1542.
Tsong, T. Y. (1976) *Biochemistry 15*, 5467.

Reconstitution of Lactic Dehydrogenase from Pig Heart after Reversible High-Pressure Dissociation†

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ABSTRACT: Lactic dehydrogenase from pig heart was reversibly dissociated to monomers at high hydrostatic pressure. As shown by equilibrium measurements making use of the yield of reactivation and high-pressure fluorescence emission spectra, the dissociation/association can be described by a consecutive dissociation/unfolding mechanism according to $N \rightleftharpoons 4M' \rightleftharpoons 4M^*$ [Müller, K., Lüdemann, H.-D., & Jaenicke, R. (1981) *Biophys. Chem.* (in press)]. The extent and rate of dissociation depend on pressure as well as on the conditions of the solvent. Maximum yields of reconstitution are achieved under anaerobic conditions, after dissociation by 20-min incubation at 1200 bar in 0.2 M Tris-HCl, pH 7.6, in the presence of 1 mM ethylenediaminetetraacetic acid and 10 mM dithioerythritol. At concentrations $c \leq 25$ μg/mL (≤0.72 μM), reconstitution amounts to ~90%. The product of reconstitution is indistinguishable from the enzyme in its initial native state, as far as its physicochemical and enzymological properties are concerned. Based on the long-term stability of the enzyme under optimum reconstitution conditions, the kinetics of reactivation and renaturation after decompression were measured at 2 μg/mL $< c <$ 25 μg/mL. The sigmoidal kinetics can be quantitatively described by an irreversible sequential uni-bimolecular mechanism, assuming inactive subunits [Jaenicke, R. (1979) *FEBS-Symp. 52*, 182–198]; the respective first- and second-order rate constants under optimum conditions of reconstitution are $k_1 = (1.5 \pm 0.1) \times 10^{-3}$ s^{-1} and $k_2 = (3.5 \pm 0.2) \times 10^3$ M^{-1} s^{-1} (20 °C). As shown by independent evidence from cross-linking experiments, the rate-limiting association reaction is the dimerization of dimers generating the native tetramer. In accordance with the above-mentioned sequential dissociation/unfolding mechanism, the sigmoidicity of the kinetic traces is more pronounced after pressure deactivation under strongly denaturing conditions. Under these conditions, the yield and rate of reactivation are decreased.

The heart isoenzyme of lactic dehydrogenase has been reversibly dissociated to inactive subunits under a variety of denaturing conditions (Teipel & Koshland, 1971; Levi & Kaplan, 1971; Chilson et al., 1966; Jaenicke, 1978). Special attention has been focused on the acid dissociation of the enzyme (Anderson & Weber, 1966; Levitzki, 1972; Jaenicke, 1974; Vallee & Williams, 1975; Rudolph et al., 1977a) since this method provides high yields of reconstitution.

In this study, the reconstitution of lactic dehydrogenase from pig heart after deactivation, denaturation, and dissociation at elevated hydrostatic pressure is reported. The influence of the conditions of high-pressure deactivation on the kinetics of reconstitution is studied with special emphasis on the com-

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