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Slow Refolding Kinetics in Yeast Iso-2 Cytochrome *c*†

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ABSTRACT: In refolding of iso-2 cytochrome *c* from *Saccharomyces cerevisiae*, there are two slow folding reactions, τ_{1a} and τ_{1b}. The slower of the slow reactions, τ_{1a} = 100–200 s, is observed only by absorbance changes, while τ_{1b} (10–20-fold faster) is detected by fluorescence changes. The temperature dependence of the rates of these reactions has been measured: for kinetic experiments ending below the folding–unfolding transition zone (pH 7.2, 0.3 M guanidine hydrochloride, 5–30 °C), the activation enthalpies are $\Delta H^{\ddagger}$ = 27 kcal/mol for τ_{1a} and 21 kcal/mol for τ_{1b}. Double-jump (unfolding, then refolding) experiments demonstrate that the two sets of species responsible for the slow folding reactions are generated slowly but at different rates under unfolding conditions (3 M guanidine hydrochloride, pH 7.2, 20 °C). Finally, as a test for changes in the population of the slow refolding species under different unfolding conditions, the amplitudes for slow refolding have been measured as a function of the initial unfolding conditions with the final refolding conditions held constant. Over the range accessible to measurement in the absence of interference from other reactions, the amplitudes for fluorescence-detected (α_{1b}) and absorbance-detected (α_{1a}) slow folding are independent of guanidine hydrochloride concentration and pH in the initial conditions. Although a full description requires a more complex explanation, many of the properties of the slow folding species are those expected for proline imide bond isomerization.

The proposal that the slow kinetic phases associated with protein folding–unfolding reactions are due entirely to isomerization of proline imide bonds (Brandts et al., 1975) is subject to a variety of experimental tests. Interpreted strictly, the isomerization hypothesis requires that the properties of slow folding reactions in proteins be identical with those of proline imide bond isomerizations. Generally, this has proved not to be the case. Even the slow folding reactions of ribonuclease A, the protein chosen for the initial tests of the hypothesis, are as often at variance as in agreement with the properties expected for proline isomerization (Nall et al., 1978; Schmid & Baldwin, 1979; Cook et al., 1979; Kim & Baldwin, 1980; Schmid, 1981, 1983; Schmid & Blaschek, 1981; Schmid et al., 1984; Lin & Bandts, 1983a–c, 1984; Nall, 1985).

The kinetic behavior of the folding reactions of horse cytochrome *c* presents a more pronounced discrepancy. In this case, it has been known for some time that chemical modification of Met-80 (a heme ligand in the native protein) specifically increases the rate of slow refolding while leaving the fast reactions unaltered (Ikai, 1971) and that removal of the iron atom eliminates slow kinetic phases entirely (Henkins & Turner, 1979). Recently, an investigation of the pH and extrinsic ligand dependence of the refolding of horse cytochrome *c* has been reported (Brems & Stellwagen, 1983). They find that an intermediate kinetic phase in refolding of

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the guanidine hydrochloride (Gdn·HCl)[1]-unfolded protein disappears during refolding at acid pH and that the presence of extrinsic heme ligands eliminates both the intermediate and the slow folding phases. In contrast, similar studies of refolding of urea-denatured cytochrome *c* in the presence of imidazole do detect slow phases, suggesting a difference in the mechanism for refolding of urea as opposed to Gdn·HCl-unfolded protein (Myer, 1984). These properties conflict with a strict interpretation of the isomerization model and suggest that if isomerization is involved it is via coupling to other conformational changes in the protein [see Brems & Stellwagen (1983)].

Here we report studies of iso-2 cytochrome *c* from yeast (*Saccharomyces cerevisiae*). This protein differs by 46% in amino acid sequence from horse cytochrome *c* but almost certainly folds to the same tertiary structure. The overall kinetic properties of folding for iso-2 are similar to those of the homologous protein from horse, suggesting that the qualitative features of protein folding reactions are conserved among homologous proteins [Nall & Landers, 1981; Krebs et al., 1983; Hollecker & Creighton, 1983; also see Brems et al. (1982)]. However, particular features of folding of iso-2 either differ from or have yet to be reported for horse cytochrome *c*. For iso-2, different kinetic phases are observed when folding is monitored by different probes of protein structure. Distinct slow phases are observed for refolding monitored by absorbance (ultraviolet or visible) as opposed to fluorescence, the fluorescence changes being complete on a time scale 10–20-fold faster than absorbance changes (Nall, 1983).

The focus of this report is on the properties of the two slow refolding reactions of iso-2 and whether these properties are those expected for reactions involving proline imide bond isomerization. Three tests are provided. First, the temperature dependence of the rates is monitored in order to compare the activation enthalpies for slow folding phases to that expected for imide bond isomerization. Second, double-jump experiments (Brandts et al., 1975; Ridge, et al., 1981) are used to monitor the rate of formation of slow refolding species under unfolding conditions. If slow folding species are due to imide isomerization, then the rate of generation of these species is expected to be slow and comparable to the rates of imide isomerization. Finally, a test is provided for shifts in the equilibrium between fast and slow refolding forms of the unfolded protein: the amplitudes for the two slow folding reactions are monitored as a function of the initial pH and guanidine hydrochloride concentration while the final conditions are held constant at 0.4 M Gdn·HCl, pH 7.2, and 20 °C.

Materials and Methods

Kinetic Measurements. Refolding rate measurements monitored by absorbance were made on a Hewlett-Packard 8450A UV/Vis spectrophotometer at 418 nm. For thermostated cuvettes, base-line stability was improved by sample stirring with a microcuvette stirrer from Instech Laboratories. The portion of the stirring apparatus in contact with the sample was covered with a thin film of Teflon tape. Base-line fluctuations appeared to be random without stirring, suggesting that the instability was due to thermal schlieren effects in combination with the unusually high *f* number of the spectrophotometer (*f*/11). There was no evidence of continuous base-line drift toward higher (or lower) absorbance, as might be expected for light scattering due to protein aggregation. Rate measurements monitored by fluorescence were made in an Aminco-Bowman spectrofluorometer using an excitation wavelength of 287 nm with fluorescence monitored at 350 nm. Sample stirring was not required for the fluorescence measurements. All measurements were in the presence of 0.1 M sodium phosphate buffer with the pH of the mixing buffer adjusted to yield the indicated pH after mixing. The final protein concentration was 5×10^{-6} M (fluorescence) or (5–11) $\times 10^{-6}$ M (absorbance). Refolding reactions were initiated by adding 10 parts buffer with low Gdn·HCl concentration to one part unfolded protein at high Gdn·HCl concentration and then mixing with an adder–mixer.

Double-jump experiments started with the protein in the absence of denaturant at pH 7.2. At time zero, an equal volume of 6.0 M Gdn·HCl containing 0.1 M sodium phosphate buffer was added and mixed, yielding a solution at pH 7.2 containing 3.0 M Gdn·HCl. At time *t*, typically 1–1000 s, a 10-fold excess of 0.03 M Gdn·HCl was added, resulting in final refolding conditions of 0.3 M Gdn·HCl, pH 7.2, with final protein concentrations of 5×10^{-6} M (fluorescence) or 11×10^{-6} M (absorbance). After the final mix, changes in absorbance (418 nm) or fluorescence (excitation at 287 nm, observation at 350 nm) were monitored.

Control experiments, in which unfolding in 3.0 M Gdn·HCl was monitored directly by absorbance changes, indicated the presence of a spurious slow unfolding phase believed to be due to aggregates induced upon lyophilization (Nall & Landers, 1981) or small amounts of contaminating reduced species. The amplitude and rate of this phase have been observed to vary with the protein preparation such that better preparations [judged by a ΔA(660 nm)/ΔA(700 nm) ratio of 1.1 or less] exhibit little or no slow unfolding phase. This phase can be removed or reduced by heating the protein at pH 7.2, 0.4 M Gdn·HCl to 65 °C for 10 min and then pre-unfolding the protein in 3.5 M Gdn·HCl and diluting out or dialyzing out the denaturant. This treatment also decreases the ΔA(660 nm)/ΔA(700) ratio. The double-jump experiments reported here yielded the same result whether or not the protein had been pre-unfolded. For the protein preparations used in these experiments, spurious slow unfolding was not detected by fluorescence regardless of whether the sample had been pre-unfolded.

Kinetic amplitude measurements have been made by stopped flow and by manual mixing. The results are reported as relative amplitudes: α_{1a} or α_{1b}, which are the changes in the observed optical parameter (fluorescence or absorbance) associated with a given kinetic phase divided by the total change for all kinetic phases (fast and slow). Relative amplitudes are obtained directly from stopped-flow data since both fast and slow phases can be detected in a single experiment. For manual mixing experiments, only the slow phases are detected. The changes associated with the slow kinetic phases have been converted to relative amplitudes by normalizing the manual mixing amplitudes obtained near neutral pH (pH 6–7.2) to the relative amplitudes measured by stopped flow at neutral pH: $\alpha_{1a} = 0.26$, $\alpha_{1b} = 0.11$ (Nall & Landers, 1981; Nall, 1983; J. J. Osterhout, Jr., and B. T. Nall, unpublished results). Other methods are as described (Osterhout et al., 1985; Nall, 1983).

Results

Slow Kinetic Phases. Figure 1A,B shows refolding of iso-2 in which the unfolded protein (initial conditions: 3.0 M Gdn·HCl, pH 7.2, 20 °C) is induced to refold by dilution of the denaturant at constant pH and temperature (final con-

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

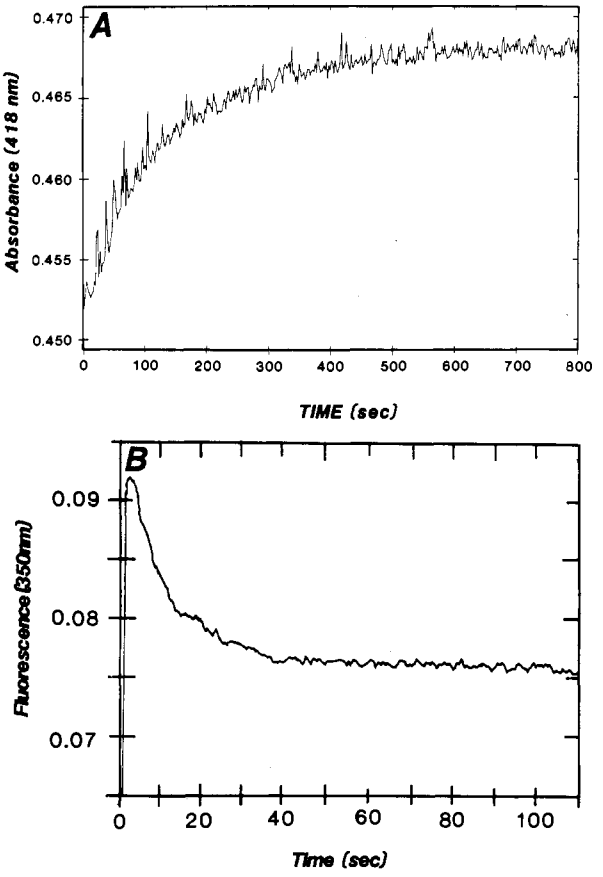


FIGURE 1: Slow kinetic phases in refolding of iso-2 cytochrome *c*. In panel A, the absorbance changes (418 nm) are shown for refolding of iso-2 following a 3.0–0.3 M Gdn·HCl concentration jump at pH 7.2. In panel B, the fluorescence changes are given at 350 nm (excitation at 287 nm) for refolding of iso-2 following a 3.0–0.3 M Gdn·HCl concentration jump at pH 7.2. Measurements are at 20 °C in the presence of 0.1 M sodium phosphate.

ditions: 0.3 M Gdn·HCl, pH 7.2, 20 °C). Refolding is monitored by absorbance (Figure 1A) and by fluorescence (Figure 1B). As reported previously (Nall, 1983), distinct kinetic phases are observed by these two different optical probes. The refolding phase monitored by fluorescence (Figure 1B) occurs on a 10–20-fold faster time scale (τ_{1b} = 10 s) than slow refolding monitored by absorbance (τ_{1a} = 145 s).

Temperature Dependence of Kinetic Processes. In further investigation of the properties of the two kinetic processes, the rates were measured over a temperature range from 5 to 30 °C. As can be seen from Figure 2, the temperature dependence of both slow refolding phases is about the same for the same final conditions: pH 7.2, 0.3 M Gdn·HCl, with the temperature restricted to a range below the unfolding transition zone. Least-squares fits to the data give a $\Delta H^{\ddagger}$ of 21 kcal/mol for refolding by fluorescence and 27 kcal/mol for refolding by absorbance at 418 nm. Within experimental errors (≈±5 kcal/mol), these activation enthalpies are in the range expected for proline imide bond isomerization (18–25 kcal/mol).

Double-Jump Kinetics. The rate of generation of the slow refolding species can be measured by double-jump methods (Brandts et al., 1975; Ridge et al., 1981) in which the protein is unfolded, held in an unfolded state for a time, *t*, and then refolded, monitoring the total amplitude of the slow refolding phases. The rationale is that if the slow kinetic phases in refolding are due to imide bond isomerization in the unfolded protein, then slow refolding species will be generated slowly as the imide bonds isomerize. For times, *t*, that are short compared to imide isomerization rates, little slow refolding

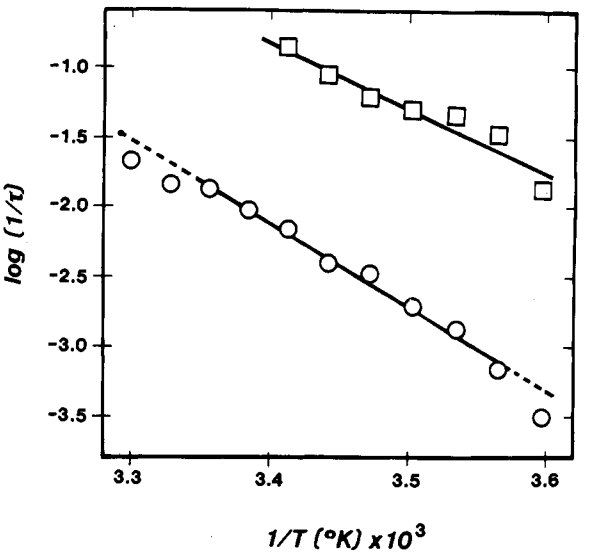


FIGURE 2: Temperature dependence of slow kinetic phases. The (base 10) logarithm of the inverse of the time constant is plotted vs. reciprocal temperature. Data for refolding monitored by absorbance, τ_{1a} (○), and by relative fluorescence, τ_{1b} (□), are given. Except for the indicated temperature, other conditions are those given in Figure 1. All points are averages of three or four independent measurements.

will be observed. For times long compared to isomerization rates, a fully developed slow phase will be observed. Intermediate values of *t* allow the rate of formation of slow refolding species to be measured and then compared to the rate expected for imide isomerization reactions.

Since there are two different slow refolding reactions for iso-2 depending on whether fluorescence or absorbance is monitored (Figure 1A,B), two experiments are required. In Figure 3A, the results of a double-jump experiment are given when absorbance changes are monitored. Figure 3B shows the results of the same experiment when fluorescence is followed. Although the experimental errors are large, the rate of buildup of slow refolding species obtained by using absorbance as an assay appears to be slower [τ_{DJ}(abs) = 114 s] than that obtained by using fluorescence [τ_{DJ}(fl) = 24 s].

Dependence of Slow Folding Amplitudes on Initial Conditions. In Figure 4, the relative amplitudes (Figure 4A) and time constants (Figure 4B) of the slow folding reactions are plotted as a function of the initial pH of the unfolded protein. Data are presented for four different (initial) concentrations of Gdn·HCl in the unfolded protein: 2, 3, 4, and 5 M. For an initial denaturant concentration of 2.0 M Gdn·HCl, stopped-flow measurements for fast refolding (τ_2) are included as well. In all cases, refolding occurs under the same final conditions: pH 7.2, 0.4 M Gdn·HCl, 20 °C. As expected for a series of kinetic experiments ending in the same final conditions, all three time constants (τ_2, τ_{1a}, and τ_{1b}) are independent of the initial pH and Gdn·HCl concentration of the unfolded protein (Figure 4B). For the conditions shown (Figure 4A), the relative amplitudes for slow folding monitored by absorbance (α_{1a}) and fluorescence (α_{1b}) are independent of Gdn·HCl concentration and pH in the initial conditions.

Above pH 7 and at initial Gdn·HCl concentrations below 5.0 M, α_{1a} shows a strong dependence on both pH and Gdn·HCl concentration (data not shown). At constant (initial) Gdn·HCl concentration, α_{1a} increases with increasing pH. At constant (initial) pH, α_{1a} decreases with increasing Gdn·HCl concentration. This is probably due to alkaline isomerization (Brandt et al., 1966; Greenwood & Palmer, 1965; Gupta & Koenig, 1971; Davis et al., 1974) of small amounts of folded protein present in the initial conditions. This reaction of the

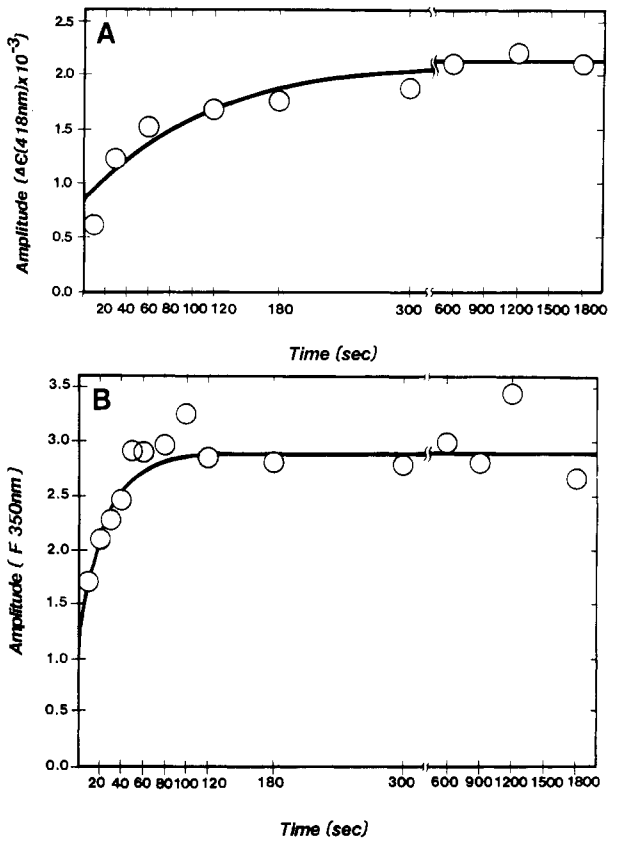


FIGURE 3: Double-jump experiments. The experiment starts with iso-2 at pH 7.2, 20 °C, in the absence of denaturant. At time zero, unfolding is initiated by adding buffer containing Gdn·HCl such that the Gdn·HCl concentration is increased to 3.0 M. At time t, refolding is initiated by diluting the Gdn·HCl to 0.3 M. The total kinetic changes associated with slow refolding are monitored by changes in absorbance ($\Delta\epsilon \times 10^{-3}$ at 418 nm) or fluorescence (relative fluorescence at 350 nm) as an assay for the amount of slow folding species generated in time t. In panel A, the rate of formation of the unfolded species responsible for phase τ_{1a} is given. The amplitudes are plotted vs. the time between initiation of unfolding and the beginning of the refolding assay. The solid line (—) is that for a single exponential of 114 s. In panel B, the rate of formation of the unfolded species responsible for phase τ_{1b} is measured. The solid line (—) is for a single exponential with a time constant of 24 s. All measurements are averages of from two to four independent measurements with both unfolding and refolding taking place at 20 °C, 0.1 M sodium phosphate, pH 7.2. The final protein concentrations are 5×10^{-6} M (fluorescence) and 11×10^{-6} M (absorbance).

fully folded protein interferes with detection of slow folding since it produces large absorbance changes in the same time range (J. J. Osterhout, Jr., and B. T. Nall, unpublished results; see Discussion). Whatever species are responsible for the strong dependence on the initial conditions, these species appear to be absent by 5.0 M Gdn·HCl where α_{1a} is no longer dependent on the initial pH (Figure 4A).

Discussion

Slow Folding Species and Imide Isomerization. The proline isomerization hypothesis (Brandts et al., 1975) provides a simple physical explanation for the observed division of structureless unfolded protein into fast and slow refolding species. The simplest form of the proposal suggests that only those chains with proline imide bonds in the native format can refold. An incorrect isomerization state of a proline is supposed to provide a complete block to structure formation and thus force the chain to remain in a random coil like state until isomerization occurs. Therefore, the kinetic and equilibrium behavior of shifts between fast and slow refolding species

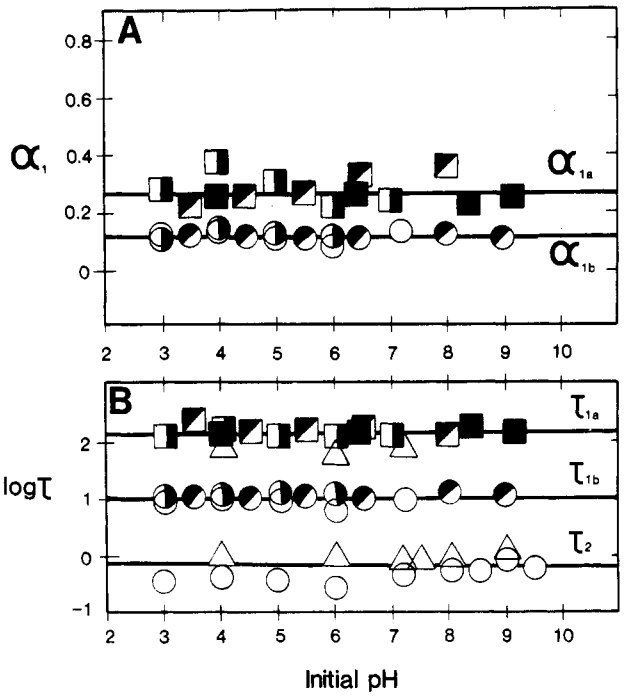


FIGURE 4: Dependence of the amplitudes and time constants on initial conditions of unfolding. The relative amplitudes (A) and time constants (B) are plotted as a function of initial pH. The protein starts in 2.0 (open symbols), 3.0 (half-filled vertical symbols), 4.0 (half-filled diagonal symbols), and 5.0 M (filled symbols) Gdn·HCl, 20 °C, at the indicated pH. Refolding is initiated by mixing such that the final conditions of refolding are 0.1 M sodium phosphate, pH 7.2, and 0.4 M Gdn·HCl, 20 °C. Fluorescence at 350 nm (○, ◑, ●), absorbance at 695 nm (△), and absorbance at 418 nm (◨, ◩, ■) are monitored. In panel A, α_{1a} is the relative amplitude for refolding monitored by absorbance changes while α_{1b} is the relative amplitude for fluorescence. In panel B, τ_{1a} is the slowest kinetic phase observed for refolding monitored by absorbance, and τ_{1b} is the slowest phase detected by fluorescence. Phase τ_2 is the fast phase in folding observed by both fluorescence and absorbance in stopped-flow mixing experiments.

should have properties similar to those of proline isomerization reactions in model peptides. Another prediction is that different physical properties used to monitor folding rates should show identical kinetic behavior. For iso-2, the former expectation is realized while the latter is not (see Figure 1A,B). As a minimum, this requires a modified isomerization model involving structured folding intermediates with distinct optical properties.

Fluorescence Monitors Formation of a Folding Intermediate. The present results confirm the previous report (Nall, 1983) that fluorescence changes detect one slow phase while absorbance changes detect another. Since refolding monitored by fluorescence appears to go to completion at least 10-fold faster than refolding monitored by absorbance, species present immediately following termination of the fluorescence-detected slow phase have properties distinct from both the native and the unfolded protein. These species will have the fluorescence properties of the native protein (since refolding is complete by fluorescence) but have nonnative absorbance properties, since absorbance-detected refolding is still in progress. One possibility is that nonnative proline imide bonds slow down re-formation of the fully native protein without providing an absolute block to structure formation. Thus, imide isomerization may provide a kinetic trap allowing transient formation of highly structured folding intermediates and nativelike species (Cook et al., 1979). While this is at odds with a strict interpretation of the proline model which does not allow for structural intermediates, this modified form of the hypothesis provides an explanation for the division of the unfolded states

of iso-2 into fast and slow refolding species without having to explain the detailed (and complex) behavior of the refolding process.

Slow Refolding Species Generated by Different Processes. The species responsible for the refolding phases τ_{1a} and τ_{1b} are distinguishable by having different rates of formation under unfolding conditions (Figure 3A,B). Thus, these species are generated by different processes—perhaps involving the isomerization of distinct proline residues. While the rates are compatible with imide isomerization, other slow reactions among unfolded species cannot be ruled out. Heme–ligand exchange reactions are an attractive alternative, but it is not clear that ligand exchange rates would be in a sufficiently slow time range. Furthermore, the initial pH independence of the amplitudes argues against heme ligation as an explanation for the generation of slow folding species. The heme–ligand state of the unfolded protein changes dramatically between acid and neutral pH (high-spin to low-spin heme–ligand complex) without detectable changes in the amplitudes of either of the slow refolding phases (Figure 4A).

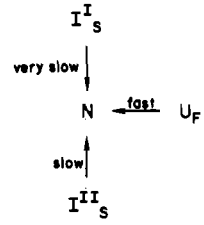
For cytochrome *c* from horse, double-jump experiments using absorbance changes as an assay have been successful in measuring the rate of formation of slow refolding species at 5 °C (Ridge et al., 1981). The relationship between these species and those of the homologous iso-2 is unclear since distinct sets of slow folding species (distinguishable by their respective optical properties) either do not exist or have not been reported for the horse protein.

Fluorescence-Detected Slow Refolding Is Complex. If the properties of the slow kinetic phases are governed by imide isomerization alone, then the relative amplitudes for slow refolding can be predicted (Brandts et al., 1975) from the time constant for refolding in combination with the time constant for generation of the slow refolding material (from double-jump experiments). Such calculations assume that rates of imide isomerization are independent of one another and of the concentration of protein denaturants. The relevant equation is

$$\alpha_1 = 1 - \tau_{DJ}/\tau_F$$

where α_1 is the relative amplitude for slow refolding, τ_{DJ} is the time constant for the generation of the slow refolding species measured by a double-jump experiment, and τ_F is the refolding time constant measured under conditions where refolding goes to completion. For folding–unfolding monitored by absorbance changes, the agreement is good. The data in Figure 1A and 3A give $\alpha_1 = 0.21$ compared to values measured directly by stopped flow in the range of 0.2–0.3 (Nall & Landers, 1981; Nall, 1983). For fluorescence, this simple model breaks down. α_1 is predicted to be zero (actually, less than zero), while the measured value is 0.1–0.15 (Nall, 1983). This apparent discrepancy may be due to the large errors in obtaining a time constant from double-jump experiments monitored by fluorescence, where the time constant (24 s) is of the order of the time required for double mixing (15–20 s). More likely, the simple analysis presented here does not apply to fluorescence-detected folding–unfolding. This reaction is known to be complex since it involves a folding intermediate with properties distinct from both the native and the unfolded protein.

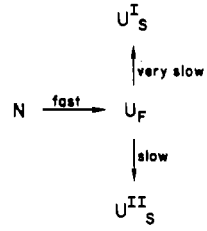
Mechanism of Folding–Unfolding. Three kinetic phases have been detected in refolding, τ_{1a}, τ_{1b}, and τ_2. Thus, a minimal mechanism requires four species and must explain the following: (1) The two slow folding phases are detected by distinct optical probes, fluorescence (τ_{1b}) or absorbance (τ_{1a}). (2) The rates of generation of the species responsible for the two slow folding reactions differ. The unfolded species which are formed more rapidly (fluorescence-detected species) also refold more rapidly, and those formed more slowly (absorbance-detected species) refold more slowly. (3) The fast phase is detected by both fluorescence and absorbance. A mechanism consistent with the refolding data is



(1)

where U_F are the fast folding species, I^I_S are the absorbance-detected slow folding species, and I^{II}_S are the fluorescence-detected slow folding species. The U_F species have the absorbance and fluorescence properties of the unfolded protein. I^I_S are species with the fluorescence of the native protein but the absorbance of the unfolded protein. I^{II}_S are species with the fluorescence of the unfolded protein but the absorbance of the native protein. Both "I" species are formed during the mixing time of the stopped-flow experiment and give rise to (undetected) changes in fluorescence (I^I_S) or absorbance (I^{II}_S). The species present immediately following mixing can be calculated from refolding amplitudes. I^I_S is estimated to be 19%, I^{II}_S is 8%, and U_F is 73% of the nonnative species.

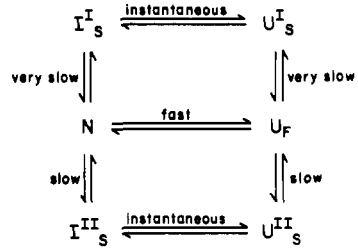
Three kinetic phases are observed for unfolding. A fast phase is detected by both fluorescence and absorbance changes (Nall, 1983; Nall & Landers, 1981) while two distinct slow phases are detected by double-jump assays, using either absorbance (Figure 3A) or fluorescence (Figure 3B) for the final refolding assay. A minimal mechanism consistent with the unfolding data is



(2)

where U_F are the fast folding unfolded species, and U^I_S and U^{II}_S are unfolded species responsible for the absorbance-detected or fluorescence-detected slow folding phases, respectively. The relative populations of the species present after unfolding has gone to completion are estimated to be 19% U^I_S, 8% U^{II}_S, and 73% U_F.

Combining eq 1 and 2 gives the overall mechanism:



(3)

where "instantaneous" is used to indicate reactions faster than the mixing time of the stopped-flow instrument (2–5 ms).[2]

[2] The $I_S \rightleftharpoons U_S$ reactions might occur in the same time range as the $N \rightleftharpoons U_F$ reaction, with all three processes giving rise to (refolding) signal changes that closely approximate a single exponential.

Mechanism 3 is certainly not unique but is one of the simplest kinetic mechanisms consistent with the data. Previously (Nall, 1983), we proposed a linear mechanism for slow folding where the product of the fluorescence-detected slow folding phase was a structural intermediate with the fluorescence of the native protein but the absorbance of the unfolded protein. The present results provide support for a parallel slow folding mechanism and suggest the presence of an additional structural intermediate with the absorbance of the native protein but the fluorescence of the unfolded protein.

Is There Residual Structure in Unfolded Iso-2? To test for residual structure in unfolded iso-2, we have carried out a series of experiments designed to detect shifts in equilibria between kinetically distinguishable unfolded species (Garel & Baldwin, 1975). The results presented in Figure 4A,B describe refolding experiments in which the initial conditions of unfolding are varied (both Gdn·HCl concentration and pH) while the final refolding conditions are held constant. The method is based on the fact that the time constants, depend only on the final conditions of the experiment while amplitudes of kinetic phases depend on both the initial and final conditions. Thus, for a series of experiments with constant final conditions but variable initial conditions (Figure 4), a change in the relative amplitudes would prove the existence of a perturbable equilibrium in the initial conditions [see Garel & Baldwin (1975)]. The results presented show that the fraction of the unfolded species which gives rise to the absorbance- or fluorescence-detected slow phases is independent of initial pH and denaturant concentration. This is the result expected if the slow folding species are generated by imide isomerization equilibria in the unfolded protein since isomerization is expected to be independent of both pH and Gdn·HCl concentration (Nall et al., 1978; Schmid & Baldwin, 1979; Garel, 1980).

In similar experiments not shown in Figure 4, refolding starting at 2.0–4.0 M Gdn·HCl and pH 8–10 showed a strong dependence of α_{1a} on the initial conditions. This is probably due to additional (interfering) absorbance changes from small amounts of folded iso-2 undergoing the alkaline isomerization reaction of the folded protein (Osterhout et al., 1985; Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974). The fact that α_{1b} does not change is consistent with this interpretation since the alkaline isomerization reaction does not produce changes in fluorescence (J. J. Osterhout, Jr., unpublished results) and thus does not interfere with fluorescence-detected refolding. On the other hand, a shift in the equilibrium between the absorbance-detected fast and slow refolding species (at high pH and below 5 M Gdn·HCl) cannot be ruled out. A change in the heme–ligand complex for the unfolded protein does occur at high pH (Osterhout et al., 1985), but there is no evidence that this change perturbs the equilibrium between the fast and slow folding species. If residual structure does shift the equilibrium toward the (absorbance-detected) slow folding species, this structure is sensitive to Gdn·HCl and has been melted out by 5.0 M Gdn·HCl.

Conclusions

The present results show that the unfolded species responsible for the two slow folding phases of iso-2 are produced at different rates under unfolding conditions. The equilibrium between both sets of slow folding species and the fast folding species is independent of Gdn·HCl concentration and pH over a wide range of conditions. Proline imide isomerization is a reasonable explanation for the generation of the slow folding species, but other processes, in particular heme–ligand exchange reactions, cannot be ruled out. For refolding, the time range and activation enthalpies for the two slow folding phases are compatible with the isomerization hypothesis. Other properties are incompatible with a simple isomerization model. The fact that one of the slow phases is detected only by absorbance while the other is detected only by fluorescence (Nall, 1983; also see Figure 1A,B) requires the presence of structurally distinct species during refolding. In addition, the relationship between the relative amplitudes and the time constants for the fluorescence-detected species is incompatible with the simplest statement of the isomerization hypothesis.

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Registry No. Iso-2 cytochrome *c*, 9007-43-6; proline, 147-85-3.

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Molecular Aspects of Functional Differences between Alcohol and Sorbitol Dehydrogenases†

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ABSTRACT: The amino acid sequence of sheep liver sorbitol dehydrogenase has been fitted to the high-resolution model of the homologous horse liver alcohol dehydrogenase by computer graphics. This has allowed construction of a model of sorbitol dehydrogenase that provides explanations why sorbitol is not a substrate for alcohol dehydrogenase, why ethanol is not a substrate for sorbitol dehydrogenase, and what determines its specificity for polyols. An important feature of the model is that one of the ligands to the active site zinc atom is a glutamic acid residue instead of a cysteine residue, which is the corresponding ligand in the homologous alcohol dehydrogenases. This is one component of the structural change that can be related to the different substrate specificities, showing how altered enzymic activity might be brought about by structural changes of the kind that it is now possible to introduce by site-directed mutagenesis and recombinant DNA techniques.

Functionally related molecules that exhibit some degree of sequence homology have, in a number of cases, been shown also to have closely similar tertiary structures [see Creighton (1983)]. From the three-dimensional structure of one member of a group of such related molecules, plausible models can be constructed from amino acid sequences for the others by computer graphics, e.g., Bedarkar et al. (1977), Isaacs et al. (1978), Blundell et al. (1978, 1983), and Eklund et al. (1984a). This approach is now gaining increasing importance because of the rapidity with which gene sequences can be determined and the possibility of site-directed mutagenesis.

Alcohol and polyol dehydrogenases with long protein chains (around 350 residues) and with a catalytically active zinc atom are members of one such family of related molecules (Jörnvall et al., 1981). The tertiary structure of only one member, horse liver alcohol dehydrogenase (LADH),[1] is known (Eklund et al., 1976, 1981). From this structure, and the amino acid sequence of yeast alcohol dehydrogenase (YADH), a plausible model for the subunit structure of the latter was developed (Jörnvall et al., 1978). YADH exhibits 23% sequence identity to LADH. Conserved functional features of these alcohol dehydrogenase members are the ligands to the catalytic zinc atom (two cysteine residues and one histidine residue) and a hydrophobic substrate binding pocket. Other mammalian alcohol dehydrogenase sequences (Bühler et al., 1984; Hempel et al., 1984) as well as the maize sequence (MADH) (Dennis et al., 1984), which all have more positional identities to LADH, also show these conserved features.

Sheep liver sorbitol dehydrogenase (SDH) also belongs to this family of alcohol/polyol dehydrogenases (Jörnvall et al., 1981, 1984a). The amino acid sequence homologies reveal about 25% positional identities between LADH and SDH (with higher homology in long segments of the protein chain) and 20% between YADH and SDH. This enzyme has also been shown to contain the expected catalytic zinc atom, but it was simultaneously found to contain only one zinc per subunit (Jeffery et al., 1984a,b) in contrast to the two Zn atoms per subunit found in LADH (Åkeson, 1964). Another overall difference between these enzymes is the quaternary structure; SDH is tetrameric like YADH and not dimeric like LADH and MADH.

There are also significant functional differences between SDH and the alcohol dehydrogenases. SDH selectively oxidizes a secondary alcohol of sorbitol and does not oxidize primary alcohols. Alcohol dehydrogenases on the other hand oxidize a range of primary alcohols, and the broad substrate specificity of the liver enzyme extends to some secondary alcohols (Dutler & Brändén, 1981).

Using criteria to maximize sequence identities while preserving the active site zinc ligands, Jörnvall et al. (1984a) have aligned the amino acid sequence of SDH with those of the alcohol dehydrogenases. This sequence alignment clearly demonstrates that the main features of the domain structures

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[1] Abbreviations: ADH, alcohol dehydrogenase; LADH, horse liver alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase; MADH, maize alcohol dehydrogenase; SDH, sheep liver sorbitol dehydrogenase.

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