Folding/Unfolding Kinetics of Mutant Forms of Iso-1-cytochrome *c* with Replacement of Proline-71†

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ABSTRACT: Proline-71, an evolutionally conserved residue that separates two short α-helical regons, is replaced by valine, threonine, or isoleucine in at least partially functional forms of iso-1-cytochrome *c* from *Saccharomyces cerevisiae* [Ernst, J. F., Hampsey, D. M., Stewart, J. W., Rackovsky, S., Goldstein, D., & Sherman, F. (1985) *J. Biol. Chem. 260*, 13225–13236]. To assign the effects of perturbations at position 71 to steps in the process of protein folding, the kinetic properties of the folding/unfolding reactions of normal protein and the three mutant forms are compared. At pH 6.0, 20 °C, fluorescence-detected folding/unfolding kinetics are monitored below, within, and above the equilibrium transition zone by using stopped-flow mixing to perform guanidine hydrochloride concentration jumps. Three kinetic phases are detected for each of the four proteins. The fastest of these phases (τ_3) differs in rate for the wild type and mutant proteins. The remaining kinetic phases (τ_1 and τ_2) have similar rates for all four proteins over the entire range of folding/unfolding conditions. The guanidine hydrochloride dependence of the relative amplitudes of the kinetic phases is complex and is sensitive to the nature of the substituent at position 71: each of the four proteins shows differences in the fraction of folding/unfolding associated with the two fastest rate processes. The results suggest that it is the location of the mutation in the primary structure rather than the nature of the substituent that determines which kinetic step (or steps) is changed in rate. However, the kinetic amplitudes (α_2 and α_3) are very sensitive to the nature of the substituent at position 71. Thus different mutations do have different effects on the stability of the species responsible for fast folding phases. Contrary to the expectations of the proline isomerization hypothesis [Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963], replacements of Pro-71 have little effect on fluorescence-detected slow refolding. Replacement of Pro-71 by Val-71 does not affect either the amplitude (α_1) or time constant (τ_1) for slow refolding. Neither the Thr-71 nor the Ile-71 replacement alters the rate of slow refolding, although decreases in relative amplitude are observed.

We propose that mechanistic studies of the folding of mutant proteins will aid in deciphering the code relating amino acid sequence to tertiary structure and function. Attempts have been made to decipher this code by comparing amino acid sequences to static three-dimensional structures. This approach has met with limited success (Levitt & Warshel, 1975; Nemethy & Scheraga, 1977; Sternberg & Thornton, 1978). Some progress has been made in the prediction of regular recurring structures (Lim, 1974; Levitt, 1978; Chou & Fasman, 1978), but further improvements require an understanding of the "feedback" mechanism by which long-range tertiary interactions influence the choice between alternative local structures (Kabsch & Sander, 1983). Thus, advances in the theoretical treatment of any one aspect of the protein-folding problem depend on progress in other areas.

Experiments are needed that focus on the interplay between secondary and tertiary structure formation. More emphasis should be put on the process (rather than exclusively on the product) of protein folding. An approach, analogous to that used successfully for mechanistic studies of organic or enzyme-catalyzed reactions, is investigation of structure–reactivity profiles for refolding of mutant proteins. Directed mutagenesis techniques allow mutant proteins to be generated with great facility and specificity (Zoller & Smith, 1983; Zoller et al., 1983; Pielak et al., 1985; Shortle & Lin, 1985), but which of the mutant forms will aid in deciphering the folding code? Nature protects important residues in the primary structure by conserving them in families of homologous proteins. These conserved sites are known to be important for some aspect of protein translocation, processing, function, or folding and are good candidates for informative studies of mutant proteins.

Our objective is to understand how amino acid substitutions at or near conserved locations alter the process of protein folding. Answers to the following questions are sought: (1) Do perturbations differing in degree but at the same site affect the same kinetic phases? (2) Are additional kinetic processes generated in mutant proteins? (3) Can the cooperativity of the unfolding transition be disrupted by mutations so that partially folded states are populated at equilibrium? We have chosen yeast iso-1-cytochrome *c* for investigations of how mutations affect protein stability (Ramdas et al., 1986) and the kinetics of protein folding. To monitor folding or functional properties of monomeric iso-1,[1] it is necessary to block cys-

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; C_m, midpoint of the Gdn·HCl-induced unfolding transition; iso-1, iso-1-cytochrome *c* from *Saccharomyces cerevisiae*; Ile-71 iso-1, Val-71 iso-1, etc., mutant forms of iso-1 in which proline-71 is replaced by isoleucine, valine, etc.; iso-1-MS, iso-1 treated with methyl methanethiosulfonate; Val-71 iso-1-MS, Thr-71 iso-1-MS, and Ile-71 iso-1-MS, mutant forms of iso-1-MS in which proline-71 is replaced by valine, threonine, and isoleucine, respectively; iso-1-AM, iso-1 with Cys-102 blocked with iodoacetamide; τ, time constant for a reaction (reciprocal of the apparent rate constant); α, amplitude of a reaction expressed as the fraction of the total observable kinetic change associated with a particular time constant.

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teine-102. In the present investigation, methyl methanethiosulfonate (Smith et al., 1975) has been used to convert the free S–H group to a S–SCH_3 group. Both wild type and mutant forms of iso-1-MS retain the spectral properties of the unmodified proteins and no longer form disulfide-linked dimers. Here we report the kinetic properties of folding/unfolding of the normal protein and three mutant forms of iso-1-MS in which the conserved proline at position 71 is replaced by valine, isoleucine, or threonine.[2] Proline-71 separates two short α-helical segments in native cytochrome c (Dickerson & Timkovich, 1975; Takano & Dickerson, 1981a,b) and thus may be important in local folding by defining the end of one helix and the beginning of another.

Materials and Methods

Growth of yeast (*Saccharomyces cerevisiae*) and protein purification were performed as previously described (Nall & Landers, 1981; Zuniga & Nall, 1983). Preparation of derivatives of wild type and altered forms of iso-1 with methyl methanethiosulfonate was according to Ramdas et al. (1986).

Kinetic Measurements of Folding/Unfolding. Instrumentation and methods of data analysis for stopped-flow measurements of folding/unfolding kinetics have been described (Nall & Landers, 1981; Zuniga & Nall, 1983; Nall, 1983). Folding was monitored by fluorescence with excitation at 287 nm and observation at right angles to the incident light through a band-pass interference filter centered at 350 nm. For refolding monitored by reducibility, the rate of formation of (ascorbic acid) reducible species was measured by absorbance at 550 nm by using methods described previously (Nall, 1983). Refolding and reduction were initiated simultaneously by mixing an unfolded protein solution containing 2.0 M Gdn·HCl in a 1:5 ratio with 0.1 M sodium phosphate buffer, pH 6.0, containing 0.1 or 1.0 M ascorbic acid. Signals were digitized with a 14-bit A/D card from RC Electronics (Santa Barbara, CA) designed to plug into an expansion slot of an Apple II+ computer. The Computerscope software supplied by RC Electronics was used for data acquisition. Minor modifications were made to the acquisition software to allow expanded time base and dual-sweep operation. All protein solutions contained 0.1 M sodium phosphate buffer adjusted to pH 6.0. In the initial conditions the protein solutions contained 0.1 M Gdn·HCl for unfolding jumps or 2.0 M Gdn·HCl for refolding jumps. The pH and Gdn·HCl concentrations of the mixing buffers were adjusted to give the indicated final Gdn·HCl concentrations at pH 6.0. Temperature was controlled separately for the drive syringes and the observation chamber at 20 °C.

Results

Folding/Unfolding Kinetics. The dependence of the folding/unfolding amplitudes and time constants on final Gdn·HCl concentration is given for Pro-71 iso-1-MS (Figure 1), Val-71 iso-1-MS (Figure 2), Thr-71 iso-1-MS (Figure 3), and Ile-71 iso-1-MS (Figure 4). For folding experiments the protein starts in a fully unfolded state in 2.0 M Gdn·HCl. Refolding is induced by dilution of the denaturant to the indicated

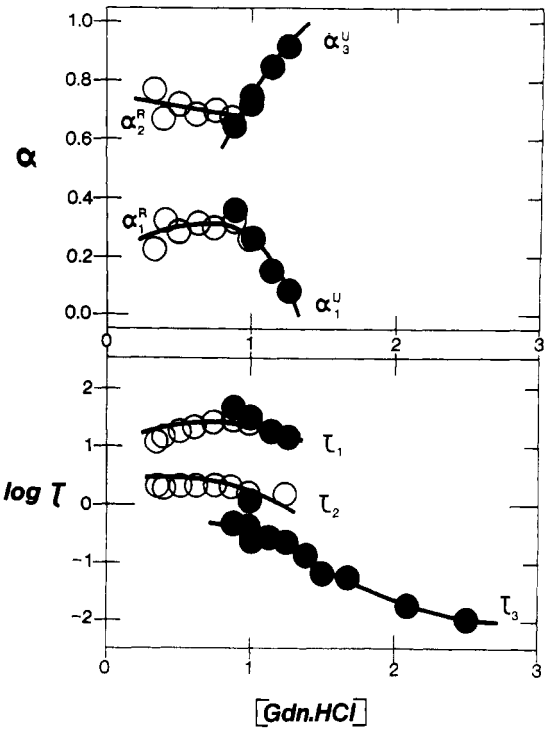


FIGURE 1: Pro-71 iso-1-MS (wild type). Gdn·HCl dependence of the relative amplitudes, α_i (top panel), and the time constants, τ_i (bottom panel), associated with fluorescence-detected phases in folding (○) and unfolding (●). The initial conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and 0.1 (for unfolding) or 2.0 M (for refolding) Gdn·HCl. Final conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and the indicated concentration of Gdn·HCl. The final protein concentration is about 5×10^{-6} M with initial protein concentrations 6-fold higher. Above 1.3 M Gdn·HCl relative amplitudes are not indicated since only one kinetic phase is detected in the stopped-flow time range.

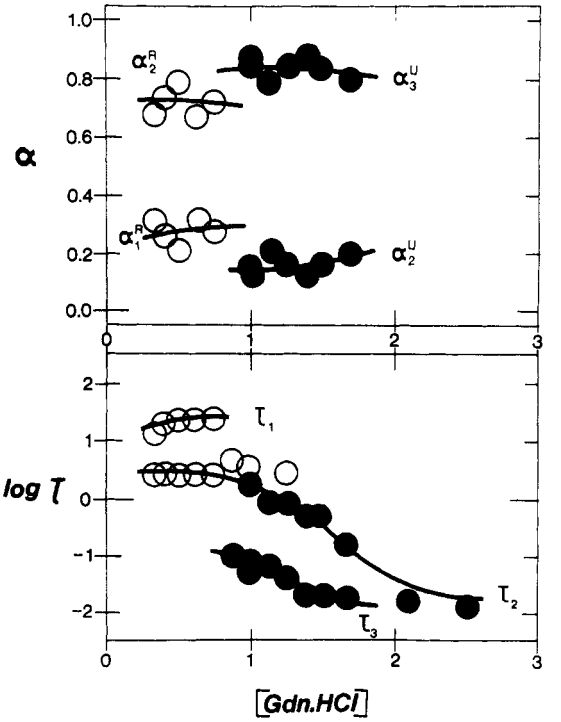


FIGURE 2: Val-71 iso-1-MS (valine replaces proline at position 71). Gdn·HCl dependence of the relative amplitudes, α_i (top panel), and the time constants, τ_i (bottom panel), associated with fluorescence-detected phases in folding (○) and unfolding (●). Other conditions are described in Figure 1. Above 2.0 M Gdn·HCl relative amplitudes are not indicated since only one kinetic phase is detected in the stopped-flow time range.

[2] The vertebrate cytochrome c numbering system is used to denote amino acid positions in order to facilitate comparison between members of the cytochrome c family. Iso-1 has five additional amino-terminal residues and one residue less on the carboxy terminus compared to vertebrate cytochromes c. Thus the numbering of iso-1 starts at position −5 and extends to position 103 [see Dickerson (1972) and Hampsey et al. (1986)]. For example, Pro-71 in the vertebrate numbering system corresponds to Pro-76 in the iso-1 numbering system.

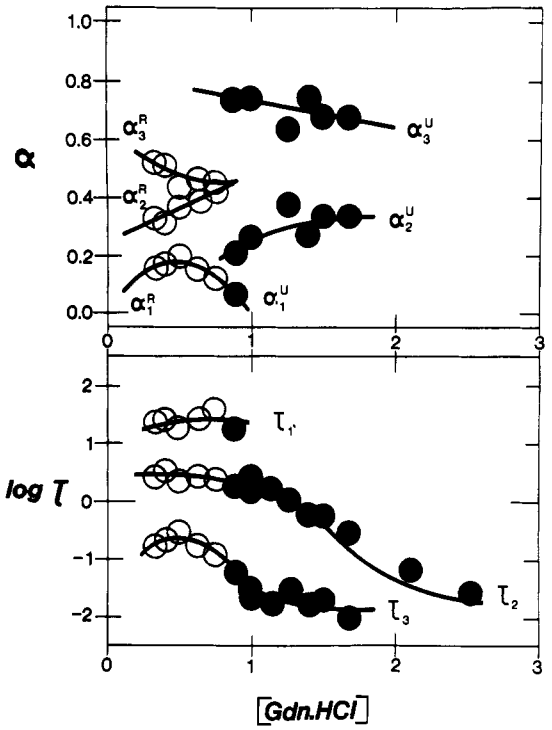


FIGURE 3: Thr-71 iso-1-MS (threonine replaces proline at position 71). Gdn·HCl dependence of the relative amplitudes, α_i (top panel), and the time constants, τ_i (bottom panel), associated with fluorescence-detected phases in folding (○) and unfolding (●). Other conditions are described in Figure 1. Above 2.0 M Gdn·HCl relative amplitudes are not indicated since only one kinetic phase is detected in the stopped-flow time range.

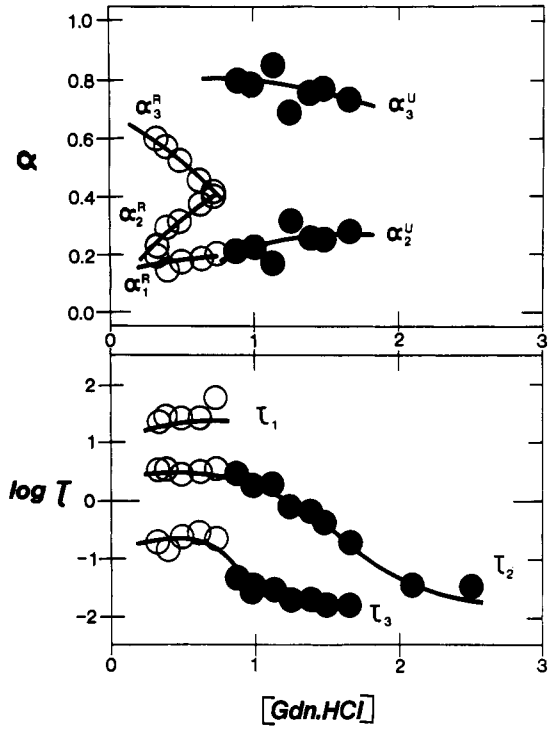


FIGURE 4: Ile-71 iso-1-MS (isoleucine replaces proline at position 71). Gdn·HCl dependence of the relative amplitudes, α_i (top panel), and the time constants, τ_i (bottom panel), associated with fluorescence-detected phases in folding (○) and unfolding (●). Other conditions are described in Figure 1. Above 2.0 M Gdn·HCl relative amplitudes are not indicated since only one kinetic phase is detected in the stopped-flow time range.

concentration by stopped-flow mixing. For unfolding experiments the protein starts in a folded state in 0.1 M Gdn·HCl, and denaturant is added by stopped-flow mixing. Three kinetic phases are detected for each of the four proteins. Compared to those of the wild type protein, two of these phases (τ_1 and τ_2) are unchanged in rate in the mutant proteins. The third (and fastest) of the three phases shows an increased rate in the mutant proteins. For all the proteins the relative amplitude behavior is complex and is sensitive to the nature of the substituent at position 71.

(*a*) *Pro-71 Iso-1-MS.* The kinetic behavior of iso-1-MS is almost the same as for iso-1-AM (Zuniga & Nall, 1983) except that different fast phases are observed for folding (τ_2) and unfolding (τ_3) of iso-1-MS. This is probably due to differences in final pH (see Discussion). The fast phases account for the major part of the amplitude in both refolding (α_2 = 0.7–0.8) and unfolding (α_3 = 0.7–1.0). A slow phase (τ_1) is detected for both folding and unfolding. For experiments ending near the middle of the transition zone the amplitude of this phase is the same for unfolding and refolding experiments ($\alpha^R{}_1 = \alpha^U{}_1$). Above the midpoint of the unfolding transition the amplitude (α_1) decreases to zero as the final (unfolding) conditions approach the upper edge of the transition zone.

(*b*) *Val-71 Iso-1-MS.* The amplitude and time constant behavior is most similar to that of the normal protein (Pro-71 iso-1-MS) when valine is substituted at position 71. Two phases, τ_2 and τ_1, have the same rates for Val-71 iso-1-MS and Pro-71 iso-1-MS over the entire Gdn·HCl concentration range in which they can be compared. Also $\alpha^R{}_2$ is the same for the two proteins. Two features of unfolding of Val-71 iso-1-MS differ from wild type: (1) The time constant for fast unfolding, τ_3, is about 5-fold faster for Val-71 iso-1-MS, and (2) two fast phases, τ_2 and τ_3, are observed in unfolding of Val-71 iso-1-MS while only τ_3 is detected for Pro-71 iso-1-MS.

(*c*) *Thr-71 Iso-1-MS.* Substantial changes in the kinetic pattern occur when threonine replaces proline at position 71. Like the valine replacement, phase τ_2 is detected in unfolding as well as refolding. However, phase τ_3 is observed for refolding experiments in addition to τ_1 and τ_2. The amplitude behavior is complex with different fast-phase amplitudes for unfolding ($\alpha^U{}_2$, $\alpha^U{}_3$) and refolding ($\alpha^R{}_2$, $\alpha^R{}_3$) experiments. For refolding, $\alpha^R{}_2$ and $\alpha^R{}_3$ are about equal (0.45) near the transition midpoint, but as the final conditions approach the pretransition region $\alpha^R{}_3$ increases while $\alpha^R{}_2$ decreases. Over the same Gdn·HCl concentration range there is little change in $\alpha^R{}_1$. It is interesting that the sum of the amplitudes ($\alpha^R{}_2 + \alpha^R{}_3$) for fast refolding of Thr-71 iso-1-MS shows essentially the same behavior as the amplitude for fast folding ($\alpha^R{}_2$) for Val-71 iso-1-MS and Pro-71 iso-1-MS. Within errors τ_1 and τ_2 are the same for Thr-71 iso-1-MS, Val-71 iso-1-MS, and Pro-71 iso-1-MS. Phase τ_3 is as much as 10-fold faster than the corresponding phase for iso-1-MS and appears to be about 2-fold faster than τ_3 for Val-71 iso-1-MS.

(*d*) *Ile-71 Iso-1-MS.* For Ile-71 iso-1-MS the behavior of all three time constants is the same as for Thr-71 iso-1-MS. The two mutant proteins have the same qualitative dependence of amplitudes on final conditions, but there are quantitative differences. Again $\alpha^R{}_3$ and $\alpha^R{}_2$ are equal (0.40) near the transition midpoint, but the Gdn·HCl concentration dependence is stronger for Ile-71 iso-1-MS: $\alpha^R{}_3$ increases from 0.4 to 0.6 and $\alpha^R{}_2$ decreases from 0.4 to 0.2 as the final conditions for refolding move away from C_m toward the pretransition base-line region. In unfolding of Ile-71 iso-1-MS, $\alpha^U{}_3$ is slightly larger and $\alpha^U{}_2$ slightly smaller than the corresponding relative amplitudes for Thr-71 iso-1-MS.

Refolding in the Presence of Ascorbic Acid. Ridge et al. (1981) have shown that refolding of cytochrome *c* in the

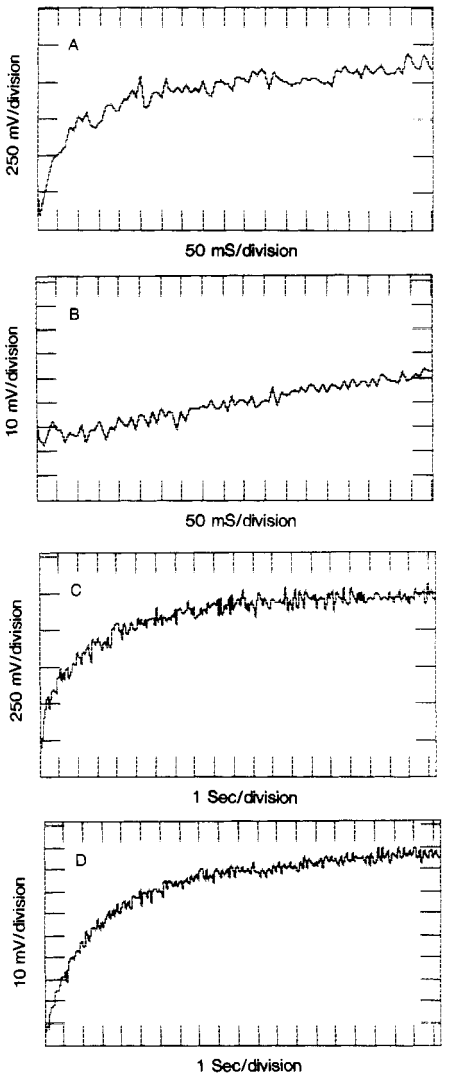


FIGURE 5: Comparison of fluorescence-detected refolding (excitation at 287 nm, detection at 350 nm) and refolding monitored by ascorbate reducibility (absorbance changes at 550 nm) for Thr-71 iso-1-MS. Phase τ_3 is detected by fluorescence (A) but not by ascorbate reducibility (B). Phase τ_2 is detected by both fluorescence (C) and ascorbate reducibility (D). Initial conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and 2.0 M Gdn·HCl. Final conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and 0.33 M Gdn·HCl. For refolding monitored by ascorbate reducibility the final conditions contain 0.83 M ascorbic acid. Final protein concentrations are about 5×10^{-6} M with initial concentrations 6-fold higher.

presence of ascorbic acid can be used to monitor the rate of formation of (ascorbic acid) reducible centers. The approach is based on the finding (Ridge, 1978) that unfolded (horse) cytochrome *c* is reduced by ascorbic acid at a rate 10^3-fold more slowly than native cytochrome *c*. Under conditions where the bimolecular rate of reduction is faster than refolding, heme reduction can be used to monitor the rate of appearance of the native protein. Previously, refolding in the presence of ascorbic acid has been used to show that the product of the fast folding phases for horse (Ridge et al., 1981) and yeast iso-2 (Nall, 1983) cytochromes *c* is native protein. In Figure 5 the rate of refolding of Thr-71 iso-1-MS measured by ascorbate reduction [$\Delta A(550)$] is compared to fluorescence-detected refolding. While phase τ_2 is detected by both reducibility and fluorescence, τ_3 is observed only by fluorescence. Thus phase τ_2 yields reducible species as a product but phase τ_3 does not. The same result is obtained for Ile-71 iso-1-MS, Val-71 iso-1-MS, and Pro-71 iso-1-MS: phase τ_2 (but not phase τ_3) produces reducible species.

Discussion

Investigations of changes in stability of mutant proteins have been reported for bacteriophage T_4 lysozyme (Hawkes et al., 1984), the α-subunit of tryptophan synthase (Matthews et al., 1980; Matthews & Crisanti, 1981; Yutani et al., 1982a,b; Ogasahara et al., 1984), and cytochrome *c* [horse (Juillerat & Taniuchi, 1986); yeast iso-1 (Ramdas et al., 1986)]. With the exception of the α-subunit of tryptophan synthase (Crisanti & Matthews, 1981; Matthews et al., 1983; Beasty & Matthews, 1985; Beasty et al., 1986) little is known about how mutations affect rates of folding/unfolding. In contrast to the α-subunit (a two-domain protein) the present investigation of normal and mutant forms of yeast iso-1-cytochrome *c* provides information on mutation-induced changes in folding/unfolding kinetics for a single-domain protein of known tertiary structure, but with a covalently attached heme. The present series of mutant proteins are all point mutations that replace Pro-71, a conserved amino acid in the primary structure of members of the mitochondrial cytochrome *c* family. Considering the complexity of the folding process, the results are surprisingly simple. Within errors (and for all three mutant proteins) only the fastest of the three kinetic phases in folding/unfolding, τ_3, exhibits mutation-induced changes in rate. The observed increases are similar for all three mutant forms, but Val-71 iso-1-MS, the least perturbed mutant protein (Ramdas et al., 1986), is increased in rate less than Thr-71 iso-1-MS and Ile-71 iso-1-MS (the comparison is confined to Gdn·HCl concentrations that are within the unfolding transition zone and above C_m, Figures 2–4).

Do Mutant Proteins Fold to a Native-like State? Although all of the proteins considered here are functional in vivo and retain properties associated with the native form of cytochrome *c* (in particular, a 695-nm absorbance band), the optical properties of the two least stable mutant proteins differ from those of wild type (Ramdas et al., 1986). Thus Thr-71 iso-1-MS and Ile-71 iso-1-MS may fold to a slightly different conformation. This may or may not require changes in the folding mechanism. Since there are no detectable differences in the spectral properties of Val-71 iso-1-MS and Pro-71 iso-1-MS, replacement of proline with valine at position 71 leads to a fully native conformation as judged by absorbance spectroscopy.

Mechanism for Folding of Pro-71 Iso-1-MS and Val-71 Iso-1-MS. Previously (Zuniga & Nall, 1983) we have investigated the folding/unfolding kinetics of iso-1-AM (Pro-71 iso-1 with cys-102 blocked by reaction with iodoacetamide) at pH 7.2. The behavior of the amplitudes and time constants was shown to be consistent with the mechanism

$$N \underset{}{\overset{\text{fast}}{\rightleftharpoons}} U_F \overset{\text{slow}}{\rightleftharpoons} U_S \qquad (1)$$

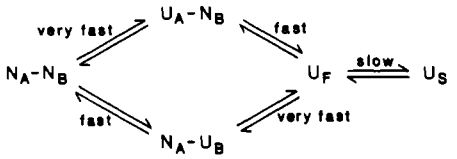
where N is the native protein and U_F and U_S are respectively fast and slow refolding forms of the unfolded protein. The folding/unfolding behavior of Pro-71 iso-1-MS at pH 6.0 is very similar to that of Pro-71 iso-1-AM at pH 7.2, with an important difference: for Pro-71 iso-1-MS distinct fast-phase rate processes are observed for unfolding (τ_3) and refolding (τ_2) while for Pro-71 iso-1-AM a single kinetic phase (τ_2) is observed for both unfolding and refolding. This is probably due to the small difference in final pH since investigation of the pH dependence of refolding for the homologous yeast iso-2 cytochrome *c* shows that a single fast phase observed at neutral pH divides into two fast phases as the pH is lowered (Nall, unpublished data). An analogous situation occurs for refolding of ribonuclease A (Hagerman & Baldwin, 1976): at acid pH there are two fast unfolding phases while at neutral pH there

is only one. A mechanism proposed by Hagerman and Baldwin (1976) for ribonuclease A is appropriate in the present case:

$$N \overset{\text{very fast}}{\rightleftharpoons} I \overset{\text{fast}}{\rightleftharpoons} U_F \overset{\text{slow}}{\rightleftharpoons} U_S \qquad (2)$$

where the I species are transient intermediates in a folding/unfolding kinetic experiment. Such species are assumed to be undetectable at equilibrium but are highly populated (transiently) in a kinetic experiment. For unfolding or refolding under limiting conditions (experiments ending above or below the transition zone) microscopic rate processes may be assigned to specific kinetic phases. In unfolding the $N \rightleftharpoons I$ reaction results in phase τ_3 as the N and I species attain a preequilibrium prior to conversion to U_F species in phase τ_2. For refolding (ending below the transition zone) the $U_F \rightleftharpoons I$ step is rate-limiting so a single fast folding phase (τ_2) is observed. Mechanism 2 provides a suitable description for the amplitude and time constant behavior for both Pro-71 iso-1-MS and Val-71 iso-1-MS [for application of mechanism 2 to the folding/unfolding kinetics of ribonuclease A, see Hagerman and Baldwin (1976)]. However, the fact that τ_2 is detected in unfolding of Val-71 iso-1-MS (but not of Pro-71 iso-1-MS) suggests that the valine substitution destabilizes the I species, thus shifting the preequilibrium more toward the N species.

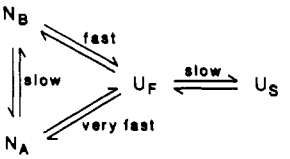
Change in Mechanism for Thr-71 Iso-1-MS and Ile-71 Iso-1-MS? When taken together, two features of the kinetic patterns for Thr-71 iso-1-MS and Ile-71 iso-1-MS are at odds with mechanism 2 [see Ikai and Tanford (1973) and Hagerman (1977)]: (1) both fast phases are observed for unfolding and refolding; (2) the amplitude of phase τ_2 is always the same sign as the amplitude of phase τ_3. The results for Thr-71 iso-1-MS and Ile-71 iso-1-MS cannot be explained by mechanism 2 since this mechanism requires that phase τ_2 and phase τ_3 have amplitudes of opposite sign (an induction phase) for either unfolding or refolding (Ikai & Tanford, 1973). Ikai (1971) discusses another mechanism that provides a good description of the present results:[3]



(3)

Mechanism 3 assumes that fast folding/unfolding involves two domans, A and B. The domains are independent of one another, but domain A unfolds faster than domain B. The two fast phases in folding/unfolding are assigned to the two-state folding/unfolding of domain A (τ_3) or domain B (τ_2). The U_F species are the direct product of unfolding of both domains. U_S species are generated from U_F species in a slow reaction, perhaps involving proline imide bond isomerization (Osterhout & Nall, 1985) as originally proposed by Brandts et al. (1975).

Mechanism 4, in which N_A and N_B are distinct *forms* of the folded protein (rather than different domains), seems more likely in the present case. This mechanism is indistinguishible from mechanism 3 in the limit where the $N_A \rightleftharpoons N_B$ equilibrium is slow compared to $N \rightleftharpoons U$ processes. The essence of mechanism 4 is a slow equilibrium between two folded species, N_A and N_B. Compared to Pro-71 iso-1-MS (and Val-71 iso-1-MS), both Ile-71 iso-1-MS and Thr-71 iso-1-MS show



(4)

decreased 695-nm absorbance (Ramdas et al., 1986). This decrease may be due to a shift in the equilibrium between native species (N_B) with a full intensity 695-nm band and native-like or alkaline species (N_A) with weak or no 695-nm absorbance (Ramdas and Nall, unpublished data). The presence of an equilibrium between multiple folded forms may explain the changes in stability and kinetic properties for these two (more highly perturbed) mutant proteins. Equilibria between folded states with variable 695-nm absorbance have been reported for horse cytochrome *c* on addition of urea (Myer et al., 1980, 1981), or on raising the pH (Greenwood & Palmer, 1965).

Tests of Kinetic Mechanisms. Mechanisms 2–4 provide some of the simplest explanations for the folding/unfolding kinetics. A minimal test of these mechanisms is given by comparing fast refolding phases monitored by ascorbate reduction to refolding phases observed by fluorescence changes. For fluorescence-detected refolding both phase τ_3 and phase τ_2 are expected for mechanisms 3 and 4 while phase τ_2 alone is expected for mechanism 2. Assuming that only fully native species (N, N_A–N_B, or N_B) are capable of reduction by ascorbate, all three mechanisms (i.e., mechanisms 2–4) predict that phase τ_2 will be the only fast folding phase with ascorbate-reducible species as a product. Experimental data verify this prediction (Figure 5).

For either of the kinetic schemes proposed in mechanisms 3 and 4 the most destabilizing substitutions at position 71 (Thr and Ile) lead to an important change in the folding process. Rather than folding through a linear sequence of states (mechanism 2), these mutant proteins fold by independent assembly of domains (mechanism 3) or fold to a state consisting of two folded forms, either of which may be generated directly from the U_F species (mechanism 4). In either case the required order of the folding process is disrupted by mutation.

Replacements of Pro-71 and Slow Folding Phases. The proline isomerization hypothesis (Brandts et al., 1975) explains slow folding kinetic phases in terms of cis–trans imide bond isomerization of proline residues. Thus replacement of a proline (in particular a conserved proline) is expected to affect the rate and amplitude of slow refolding. Thr-71 iso-1-MS and Ile-71 iso-1-MS do show a small decrease in amplitude associated with phase τ_1 (relative to wild type protein) but have identical slow folding rates. However, Val-71 iso-1-MS has the same amplitude and rate of slow folding as the wild type protein, so it is clear that isomerization of Pro-71 is not involved in the fluorescence-detected slow refolding reaction. This leads to two separate questions: (1) Why does not Pro-71 contribute to slow refolding? (2) What is the nature of the physical process that is responsible for slow refolding? Since there are three additional proline residues in iso-1 (conserved prolines at positions 30 and 76 and Pro-25 at a variable site), isomerization of one or all of these residues could generate slow kinetic phases. Nevertheless, processes other than proline isomerization should not be ruled out. An explanation for the absence of mutation-induced changes in slow folding could take several forms. One possibility is that Pro-71 remains in a native (probably trans) isomeric state in the unfolded protein and thus isomerization is not required in refolding. Retention of the native isomeric state on unfolding has been reported for

[3] The mechanism discussed by Ikai (1971) does not include the U_S species but is otherwise identical with our mechanism 3.

Pro-117 in ribonuclease A (Lin & Brandts, 1984) and explains why that residue does not contribute to slow folding. Another explanation might be that folding to (reducible) native-like conformations can occur with Pro-71 in either the cis or trans state. One final possibility is that structure in folding intermediates enhances the rate of isomerization so that isomerization of Pro-71 occurs in a fast folding reaction [see Schmid (1986)].

CONCLUSIONS

Replacements of conserved Pro-71 in iso-1-MS cytochrome *c* affect the rate of the fastest of three detectable kinetic phases associated with folding/unfolding. Two other kinetic phases in folding/unfolding of the mutant proteins do not differ significantly in rate from the analogous kinetic phases in folding/unfolding of the normal protein. Thus the effect of amino acid replacements at position 71 on the folding/unfolding kinetics is assigned to a specific kinetic phase. The behavior of the relative amplitudes associated with the kinetic phases is complex and depends on the nature of the substituent at position 71. Since replacement of Pro-71 has little or no effect on slow refolding, isomerization of Pro-71 is not involved in generation of the fluorescence-detected slow refolding species.

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Registry No. L-Pro, 147-85-3; L-Val, 72-18-4; L-Ile, 73-32-5; L-Thr, 72-19-5; cytochrome *c*, 9007-43-6.

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