Replacement of a Conserved Proline Eliminates the Absorbance-Detected Slow Folding Phase of Iso-2-cytochrome *c*†

Ladonna C. Wood,‡ Terry B. White,§ Latha Ramdas,‖ and Barry T. Nall*

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284, and Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, Texas 77225

Received April 6, 1988; Revised Manuscript Received July 13, 1988

ABSTRACT: As a test of the proline isomerization model, we have used oligonucleotide site-directed mutagenesis to construct a mutant form of iso-2-cytochrome *c* in which proline-76 is replaced by glycine [Wood, L. C., Muthukrishnan, K., White, T. B., Ramdas, L., & Nall, B. T. (1988) *Biochemistry* (preceding paper in this issue)]. For the oxidized form of Gly-76 iso-2, an estimate of stability by guanidine hydrochloride induced unfolding indicates that the mutation destabilizes the protein by 1.2 kcal/mol under standard conditions of neutral pH and 20 °C ($\Delta G°_u$ = 3.8 kcal/mol for normal Pro-76 iso-2 versus 2.6 kcal/mol for Gly-76 iso-2). The kinetics of folding/unfolding have been monitored by fluorescence changes throughout the transition region using stopped-flow mixing. The rates for fast and slow fluorescence-detected refolding are unchanged, while fast unfolding is increased in rate 3-fold in the mutant protein compared to normal iso-2. A new kinetic phase in the 1-s time range is observed in fluorescence-detected unfolding of the mutant protein. The presence of the new phase is correlated with the presence of species with an altered folded conformation in the initial conditions, suggesting assignment of the phase to unfolding of this species. The fluorescence-detected and absorbance-detected slow folding phases have been monitored as a function of final pH by manual mixing between pH 5.5 and 8 (0.3 M guanidine hydrochloride, 20 °C). Both the amplitudes and rates for fluorescence-detected slow folding are independent of pH and are indistinguishable from fluorescence-detected slow folding of normal iso-2. The usual absorbance-detected slow folding phase is absent in folding of Gly-76 iso-2, suggesting that the absorbance-detected slow folding species for normal iso-2 are generated by isomerization of the proline-76 imide bond in the unfolded protein. A new slow kinetic phase detected by absorbance changes is shown to be a conformational change between folded nativelike species and an altered mutant structure. We propose that *successful* folding of mutant proteins to altered conformations may, in general, follow the pathway to the nativelike state prior to conversion to altered three-dimensional structures via conformational changes.

Iso-1 and iso-2 cytochromes *c* provide a model system for understanding how changes in amino acid sequence can alter the kinetic features of protein folding/unfolding. The objective is to deduce the nature of the physical processes involved in rate-limiting steps from mutation-induced changes in the kinetics of folding. A first step in such an analysis is the assignment of mutation-induced perturbations of the folding process to particular kinetic phases in folding. Prolines are of special interest since many of the slow kinetic phases in protein folding are believed to be due to proline imide bond isomerization (Brandts et al., 1975). Of the naturally occurring amino acids, only prolines are known to exist as equilibrium mixtures of cis and trans (imide bond) isomeric forms in unstructured polypeptides. For native folds which accommodate only one isomeric form, isomerization must occur prior to or along with refolding, at least for that fraction of the unfolded protein molecules containing proline in a nonnative format.

A variety of approaches have been used to test the proline isomerization hypothesis. These include isomer-specific proteolysis (Lin & Brandts, 1983, 1984, 1985), catalysis of folding by prolyl isomerase (Fischer & Bang, 1985; Lang et al., 1987; Lang & Schmid, 1988), comparisons of the refolding kinetics of homologous proteins containing prolines in different numbers or locations (Brandts et al., 1977; Babul et al., 1978; Krebs et al., 1983), and analyses of the properties of slow protein folding reactions relative to imide isomerization in model peptides (Brandts et al., 1975; Nall et al., 1978; Schmid & Baldwin, 1978, 1979; Schmid, 1982). In addition, peptide synthesis has been combined with a three-fragment complementation system to investigate changes in stability of fragment complexes of horse cytochrome *c* on replacement of proline-30 with a series of amino acids (Poerio et al., 1986).

More recently, mutagenesis has been used to assign effects of replacement of specific prolines to individual kinetic phases in folding/unfolding of thioredoxin (Kelley & Richards, 1987) and of iso-1 and iso-2 cytochromes *c* (Ramdas & Nall, 1986; White et al., 1987). Three mutant forms of iso-1-MS[1] have

† Supported by grants from the National Institute of General Medical Sciences (GM 32980) and the Robert A. Welch Foundation (AQ-838). L.C.W. is a Robert A. Welch Foundation postdoctoral fellow. T.B.W. acknowledges support from the Robert A. Welch Foundation and from a National Research Service Award (GM-09979). This work was done during the tenure of an established investigatorship of the American Heart Association (B.T.N.).

* Address correspondence to this author at the Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760.

‡ Present address: Department of Biological Chemistry, University of California School of Medicine, Center for Health Sciences, 10833 Le Conte Ave., Los Angeles, CA 90024-1737.

§ Present address: Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, TX 77225.

‖ Present address: Department of Biochemistry, Baylor College of Medicine, Methodist Hospital A601, Houston, TX 77030.

0006-2960/88/0427-8562$01.50/0

been investigated in which Pro-71[2] is replaced by valine, threonine, or isoleucine. Each replacement leads to a protein less stable than the normal protein, but the mutant proteins still fold to nativelike conformations (Ramdas et al., 1986). When the kinetics of folding/unfolding are monitored by fluorescence, three phases are detected for normal and mutant proteins: two fast phases in the seconds to milliseconds time range and one slow phase in the 10-s time range. Replacement of Pro-71 has a specific effect on fluorescence-detected folding/unfolding: the fastest phase increases in rate while the remaining slower phases have the same rates in the normal and mutant proteins.

Effects of replacing Pro-71 on absorbance-detected slow folding are complex, depending on the folded conformation of the mutant protein as dictated by the conditions. For a Thr-71 mutant of iso-2, the absorbance-detected slow folding phase is absent at pH 7.2 but is evident (although with a rate slower than normal) for refolding at pH 5.5. Thus, the lack of absorbance-detected slow folding at higher pH is not due simply to absence of Pro-71. An explanation is that folding at low pH proceeds to a nativelike conformation via the absorbance-detected slow folding reaction but folding at higher pH is to a mutant conformation and proceeds by a pathway that lacks the slow process. This hypothesis is supported by direct observation of a pH-dependent equilibrium ($pK = 6.6$) between two fully folded conformations of Thr-71 iso-2 (White et al., 1987). Moreover, well above neutral pH, a similar pH-induced conformational change ($pK = 8.45$) occurs for normal iso-2 (Osterhout et al., 1985; L. Ramdas, unpublished results), and folding of wild-type iso-2 to the alkaline conformation also proceeds in the absence of absorbance-detected slow folding (Nall et al., 1988). These results show that loss of a slow folding phase on replacement of a proline is not sufficient evidence for assignment of a particular proline to a specific slow phase. In addition, it is necessary to show that the mutant protein folds to a nativelike conformation along a pathway that normally requires a slow folding reaction.

We present an analysis of the kinetic properties of folding/unfolding of a mutant form of iso-2 cytochrome *c* in which Pro-76 is replaced by glycine (Gly-76 iso-2). Proline is found at position 76 in all known mitochondrial cytochromes *c*. X-ray crystallography shows that Pro-76 occurs as the trans imide isomer near the protein surface and is the second amino acid in a type II reverse turn (Takano et al., 1977; Takano & Dickerson, 1981a,b; Louie et al., 1988; G. D. Brayer, unpublished results). The turn is positioned between a short segment of helix (residues 70–75) and the heme ligand,

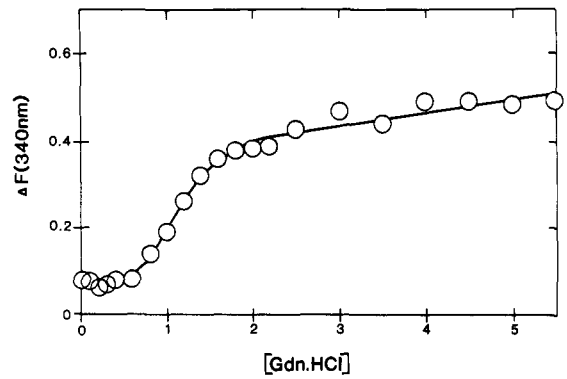


FIGURE 1: Gdn·HCl-induced equilibrium unfolding transition for Gly-76 iso-2 cytochrome *c*. Fluorescence of the protein solutions (measured at 340 nm with excitation at 280 nm) relative to that of an equimolar concentration of *N*-acetyltryptophanamide is plotted versus Gdn·HCl concentration. Conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and 5×10^{-6} M Gly-76 iso-2. For the two-state thermodynamic analysis, the base line for the native protein was assumed to be 0.07 and independent of Gdn·HCl concentration. The base line for the unfolded protein, obtained from a linear least-squares fit to the data above 1.8 M Gdn·HCl, has an intercept of 0.34 at 0 M Gdn·HCl and a slope of 3.0×10^{-2} M^{-1}.

Met-80, suggesting a structural role in aligning Met-80 for heme ligation. Gly-76 iso-2, like Thr-71 iso-2, folds to a nativelike conformation at pH 6.0 but to a mutant conformation above neutral pH. Many of the mutation-induced changes in folding/unfolding of Gly-76 iso-2 are similar to those observed for replacements of Pro-71 in iso-1-MS and iso-2. There is, however, an important difference. In contrast to replacements of Pro-71, folding of Gly-76 iso-2 to both mutant and *nativelike* conformations proceeds in the absence of the absorbance-detected slow folding reaction. The simplest explanation for the lack of the slow phase is that the absorbance-detected slow folding species of normal iso-2 are generated by isomerization of the Pro-76 imide bond.

Materials and Methods

Construction of the Gly-76 iso-2 mutant protein, growth of yeast (*Saccharomyces cerevisiae*), and protein purifications were performed as described in Wood et al. (1988). Gdn·HCl-induced equilibrium unfolding transitions were monitored by fluorescence according to Ramdas et al. (1986). Fluorescence of 5×10^{-6} M protein solutions was measured at 340 nm (excitation at 280 nm) at 20 °C, 0.1 M sodium phosphate, pH 6.0. The fluorescence of the protein solutions containing Gdn·HCl was measured relative to Gdn·HCl-containing blanks and standards containing Gdn·HCl plus 5×10^{-6} M *N*-acetyltryptophanamide. Procedures and equipment used for stopped-flow and manual mixing measurements of the kinetics of folding/unfolding have been described elsewhere (Ramdas & Nall, 1986; Osterhout & Nall, 1985; Nall, 1983; Nall & Landers, 1981). Except where indicated otherwise, kinetic measurements were at 20 °C, 0.1 M sodium phosphate, pH 6.0.

Results

Equilibrium Unfolding of Gly-76 Iso-2. In Figure 1, changes in relative fluorescence for Gly-76 iso-2 are shown for solutions containing increasing concentrations of Gdn·HCl. The fluorescence increases as the Gdn·HCl concentration increases, in accord with the suggestion of Tsong (1974) that fluorescence quenching of the single tryptophan residue by the heme is less efficient as the protein unfolds and the average distance between the heme and the tryptophan increases. These results are similar to those obtained previously for

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; iso-2, iso-2-cytochrome *c* from *Saccharomyces cerevisiae*; Gly-76 iso-2, Thr-71 iso-2, and Thr-30 iso-2, mutant forms of iso-2 in which proline-76, proline-71, or proline-30 is replaced by glycine or threonine; iso-1-MS, iso-1-cytochrome *c* from *Saccharomyces cerevisiae* treated with methyl methanethiosulfonate; Val-71 iso-1-MS, Thr-71 iso-1-MS, Ile-71 iso-1-MS, and Ser-71 iso-1-MS, mutant forms of iso-1-MS in which proline-71 is replaced by valine, threonine, isoleucine, or serine, respectively; τ, time constant of 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.

[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 iso-2 has nine additional amino-terminal residues compared to vertebrate cytochromes *c*. Both iso-1 and iso-2 have one residue less on the carboxy terminus. Thus, the vertebrate numbering of iso-1 and iso-2 starts at positions −5 and −9, respectively, and extends to position 103 [see Dickerson (1972) and Hampsey et al. (1986)]. For example, Pro-76 in the vertebrate numbering system corresponds to Pro-85 in the iso-2 numbering system.

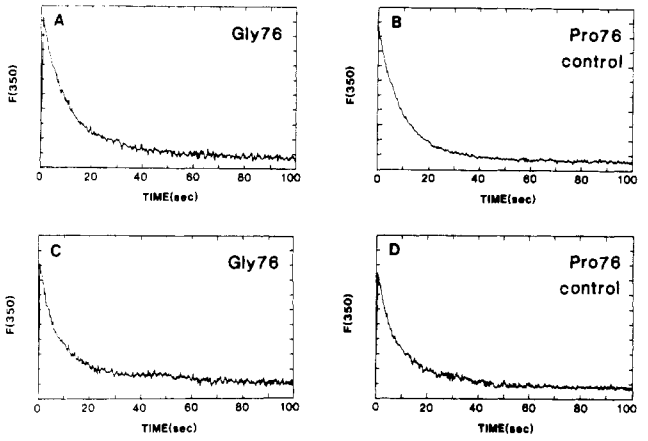


FIGURE 2: Fluorescence-detected slow folding for Gly-76 iso-2 and normal (Pro-76) iso-2 at pH 6 (A, B) and pH 7.2 (C, D). Fluorescence changes are monitored at 350 nm (excitation 287 nm) following a 10-fold dilution with buffer of a 200 × 10^{-6} M protein solution in 3.0 M Gdn·HCl and 0.1 M sodium phosphate. Initial and final pHs are the same: pH 6.0 (A, B) or pH 7.2 (C, D). Final refolding conditions are 20 °C, 0.1 M sodium phosphate, 0.3 M Gdn·HCl, and 20 × 10^{-6} M protein. Dilutions were performed manually in the fluorescence cell using an adder–mixer.

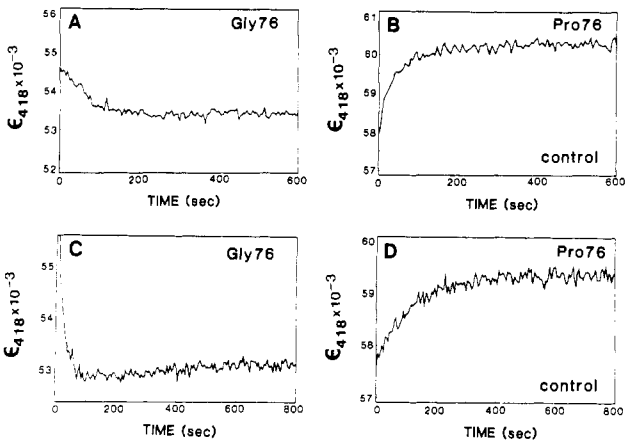


FIGURE 3: Slow refolding kinetics of Gly-76 iso-2 (mutant) and Pro-76 iso-2 (wild type) measured by absorbance changes at 418 nm. Refolding is measured at pH 6.0 (A, B) and pH 7.2 (C, D). At pH 6.0, both the mutant and the normal proteins fold to predominantly nativelike conformations. At pH 7.2, the normal protein folds to a native conformation, but Gly-76 iso-2 folds to a mutant (or alkaline) conformation. Other conditions are described in Figure 2.

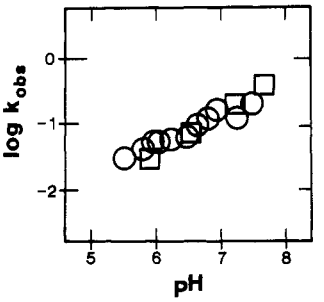


FIGURE 4: Comparison of the pH dependence of the rate of a conformational change between two folded states and the pH dependence of a slow absorbance-detected reaction that follows refolding of Gly-76 iso-2. The rate of the pH-induced conformational change (○) is measured by pH jumps in the presence of 0.3 M Gdn·HCl and 0.1 M sodium phosphate, starting at pH 5.5 (nativelike conformation) or pH 8 (mutant conformation) and ending at the indicated final pH. For initial pH 5.5, data are collected at final pH values between pH 6.0 and 7.5. For an initial pH 8.0, the final pH ranged between pH 5.5 and 6.6, overlapping with the "up" pH jump experiments. As expected for a reversible reaction, rates depend only on the final pH. The pH dependence of the rate of the slow phase that follows refolding (□) is measured by jumps in both the Gdn·HCl concentration and pH. Initial conditions for the unfolded protein are 100 × 10^{-6} M Gly-76 iso-2, 3.0 M Gdn·HCl, and 0.1 M sodium phosphate, pH 6.0. Refolding is induced by a 10-fold dilution with buffer, giving final refolding conditions of 10 × 10^{-6} M protein, 0.3 M Gdn·HCl, 0.1 M sodium phosphate, and the indicated final pH. All rates are monitored by absorbance changes at 418 nm at 20 °C.

normal (Pro-76) iso-2 except that the unfolding transition is shifted to lower Gdn·HCl concentration for the mutant protein. Assuming two-state unfolding at equilibrium, analysis of the transition according to Schellman (1978) gives a transition midpoint C_m = 1.08 ± 0.1 M/L and $\Delta G°_u$ = 2.61 ± 0.3 kcal/M. This can be compared to values of C_m = 1.49 ± 0.1 M/L and $\Delta G°_u$ = 3.8 ± 0.5 kcal/M for iso-2 under similar conditions: 20 °C, 0.1 M sodium phosphate, pH 7.2 (Osterhout et al., 1985; White et al., 1987). There is no significant change in the cooperativity of the transition as measured by the parameter $-RT\Delta b_{23}$ (Schellman, 1978). For Gly-76 iso-2, $-RT\Delta b_{23}$ = 2.4 kcal·L/M^2, and for (Pro-76) iso-2, $-RT\Delta b_{23}$ = 2.6 kcal·L/M^2 with estimated errors of ±0.1 kcal·L/M^2.

Fluorescence-Detected Slow Folding. The conformation of the folded form of Gly-76 iso-2 is known to depend on pH [see Figure 2 of Wood et al. (1988)] with an apparent pK = 6.71 for the conformational transition between the nativelike and mutant forms (L. Ramdas, unpublished results). Thus, the conformation of Gly-76 iso-2 changes from a predominantly nativelike conformation at pH 6 to a mutant conformation at pH 7.2. Between pH 6.0 and pH 7.2, normal iso-2 retains a native conformation as indicated by the presence of an intense 695-nm absorbance band (Osterhout et al., 1985). In Figure 2, the fluorescence-detected slow phases in refolding of mutant Gly-76 iso-2 and normal iso-2 are compared for experiments ending at pH 6 and 7.2. The amplitudes and rates for the fluorescence-detected slow phases are unaffected by replacement of Pro-76 by glycine and are independent of whether Gly-76 iso-2 folds to the nativelike (pH 6) or mutant (pH 7.2) conformation.

Absorbance-Detected Slow Folding. In Figure 3, absorbance-detected slow folding is compared for Gly-76 iso-2 and normal iso-2 at both pH 6 and pH 7.2. For the normal protein, slow folding phases are detected at both final pH values (Figure 3B,D). For Gly-76 iso-2, there is no evidence of absorbance changes in the direction expected for refolding to the nativelike (pH 6) or the mutant (pH 7.2) conformations. A new kinetic phase, however, is detected in the same time range as the slow folding phase (Figure 3A,C). The absorbance changes for the new phase are opposite in sign to those expected for refolding (compare Figure 3A to Figure 3B and Figure 3C to Figure 3D), and the amplitude and rate appear to be strongly pH dependent (Figure 3A vs Figure 3C). To test if the new phase results from the (pH-dependent) equilibration between the mutant and the nativelike conformations, pH jumps in the presence of 0.3 M Gdn·HCl are performed throughout the transition zone for the pH-induced conformational change. For comparison, the pH dependence of the rate of the slow process following refolding of Gly-76 iso-2 is measured over the same pH range. The results are presented in Figure 4 and show that the rates and the pH dependence of the rates are the same for the pH-induced conformational change and the slow phase that follows refolding. This supports the view that the slow absorbance changes are due to reequilibration between the two folded conformations, nativelike and mutant, following refolding of Gly-76 iso-2.

Gdn·HCl Dependence of Fluorescence-Detected Folding/Unfolding. To investigate mutation-induced changes in the

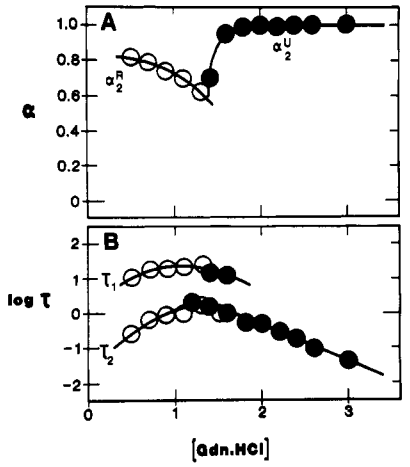


FIGURE 5: Gdn·HCl concentration dependence of (A) the relative amplitude, α_2, for the fast phase and (B) time constants, τ, for fast and slow phases in fluorescence-detected folding (○) and unfolding (●) of Pro-76 iso-2 (wild type). Fluorescence is measured at 350 nm (excitation 287 nm). Experiments are carried out by stopped-flow mixing of protein solutions with Gdn·HCl containing buffer solutions at a ratio of 1:5. Initial conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, and 0.1 M (for unfolding) or 3.0 M (for refolding) Gdn·HCl. Final conditions are 0.1 M sodium phosphate, pH 6, 20 °C, and the indicated concentration of Gdn·HCl. The final protein concentration is about 5×10^{-6} M with initial concentrations 6-fold higher. The solid lines (—) through the relative amplitude and time constants have no theoretical significance.

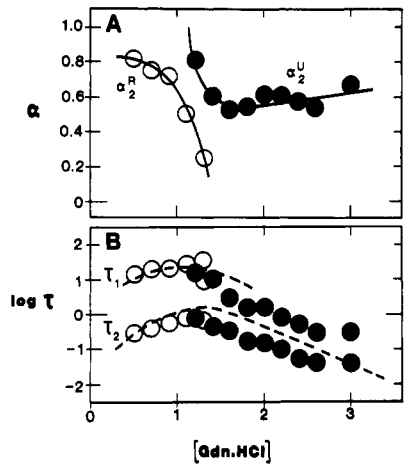


FIGURE 6: Gdn·HCl concentration dependence of (A) the relative amplitude, α_2, for the fast phase and (B) time constants, τ, for fast and slow phases in fluorescence-detected folding (○) and unfolding (●) of Gly-76 iso-2 (mutant). For comparison, the dashed lines (– – –) show the time constant behavior for normal iso-2 (from Figure 5). The solid line through the relative amplitude has no theoretical significance. Conditions are the same as described in Figure 5.

fast phases in folding/unfolding, stopped-flow mixing is used to perform Gdn·HCl jumps throughout the unfolding transition zone. Amplitudes and rates are compared as a function of the final Gdn·HCl concentration for both unfolding and refolding of normal iso-2 (Figure 5) and Gly-76 iso-2 (Figure 6). For iso-2 (Figure 5), the kinetic pattern is essentially the same as that reported previously for folding/unfolding at slightly higher pH (Nall, 1983). For Gly-76 iso-2, both fast (τ_2) and slow (τ_1) refolding reactions (Figure 6) occur with the same rates as normal iso-2 over the entire range of Gdn·HCl concentrations in which refolding is measured. Although the relative amplitudes for fast refolding (α_2^R) differ within the transition zone, the limiting behavior at low Gdn·HCl concentration is similar, extrapolating to 0.8–0.9 at zero Gdn·HCl concentration for both iso-2 and Gly-76 iso-2.

Table I: Initial pH Dependence of Fluorescence-Detected Unfolding of Iso-2 and Gly-76 Iso-2[a]

protein	initial pH	α_A	τ_A (s)	α_2	τ_2 (s)
(Pro-76) iso-2	6.0	0		1.0	0.507
Gly-76 iso-2	6.0	0.39	1.7	0.61	0.14
(Pro-76) iso-2	8.0	0.17	1.9	0.83	0.54
Gly-76 iso-2	8.0	1.0	1.5	0	
(Pro-76) iso-2	10.0	1.0	1.8	0	

[a] The experiments combine pH and Gdn·HCl jumps by stopped-flow mixing at 20 °C. Initial conditions include 30×10^{-6} M iso-2 or Gly-76 iso-2 in 0.1 M Gdn·HCl and 0.1 M sodium phosphate adjusted to the indicated initial pH. Unfolding is induced by 1:5 mixing with buffered Gdn·HCl-containing solutions to give final unfolding conditions of 5×10^{-6} M iso-2 or Gly-76 iso-2 in 2.0 M Gdn·HCl and 0.1 M sodium phosphate, pH 6.0. Relative amplitudes, α, and time constants, τ, are given for unfolding reactions for the alkaline (or mutant) species (α_A, τ_A) and the nativelike species (α_2, τ_2).

For unfolding, an increase in the rate of phase τ_2 by approximately 3-fold is observed for Gly-76 iso-2 compared to normal iso-2 over the entire Gdn·HCl concentration range in which rates can be measured. In addition, while unfolding of iso-2 occurs as a single rate process, a second slower phase is observed in unfolding of Gly-76 iso-2. Since the folded state of Gly-76 iso-2 at pH 6 is known to be an equilibrium mixture between nativelike (84%) and mutant (16%) species, the slower phase in unfolding may be unfolding from the mutant conformation, while the faster unfolding reaction is unfolding from the nativelike reaction. The pH dependence of the equilibrium between the nativelike and mutant folded conformations of Gly-76 iso-2 (pK = 6.71; L. Ramdas, unpublished results) allows this hypothesis to be tested by unfolding experiments in which the initial conditions (pH) are varied while the final unfolding conditions are held constant. Results are presented in Table I. For unfolding of Gly-76 iso-2 starting at pH 6, where both nativelike and mutant species are present in the initial conditions, two phases are detected: a major fast reaction and a slower reaction with lower amplitude. For unfolding of Gly-76 iso-2 starting at pH 8, where greater than 95% of the species are expected to be in the mutant conformation, only the slower unfolding reaction is detected.

For normal iso-2, a similar conformation change between folded species is observed at high pH (pK = 8.45; L. Ramdas, unpublished results; Osterhout et al., 1985). As expected, unfolding of iso-2 occurs in a single fast kinetic phase when unfolding is initiated at pH 6, but for experiments starting at pH 8.0 unfolding is biphasic, showing an additional slower phase. By pH 10, where more than 97% of species start in the mutant (or alkaline) conformation, unfolding of iso-2 proceeds in a single (slow) phase.

Discussion

Proline residues most likely to play key roles in folding are those that are retained in a family of homologous proteins. Figure 7 shows the locations of the three conserved and two variable proline residues in folded iso-2. Table II summarizes the slow folding behavior of the two normal yeast iso cytochromes *c* and the mutant forms in which conserved prolines have been replaced. Pro-71, between two short helical segments, may play the role of a helix breaker. Effects of replacements of Pro-71 on conformation, stability toward Gdn·HCl-induced unfolding, and folding/unfolding rates have been investigated in detail (Ramdas et al., 1986; Ramdas & Nall, 1986; White et al., 1987). Pro-30, buried in the protein interior between two sharp bends, is important in maintaining the orientation of the heme ligand, His-18. Replacement of Pro-30 with threonine produces a mutant cytochrome *c* that

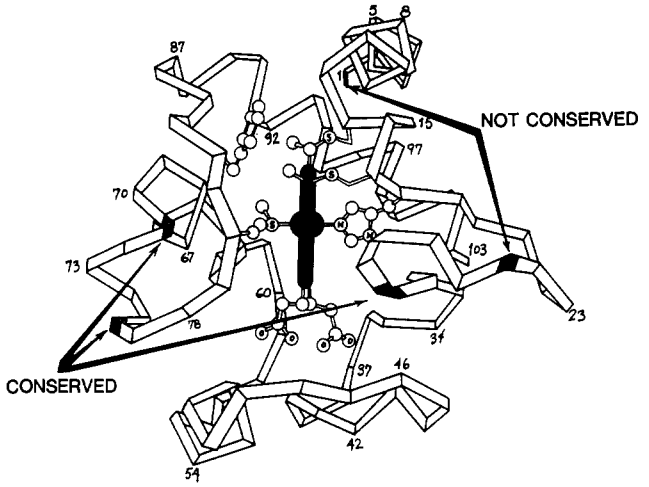


FIGURE 7: Locations of prolines in iso-2 cytochrome *c*. The positions in mitochondrial cytochromes *c* at which proline is always found are labeled *conserved*. Of the two sites that are not conserved, both iso-1 and iso-2 contain proline at position 25. The proline at position −1 in iso-2 is replaced by alanine in iso-1. The ribbon schematic of the cytochrome *c* tertiary structure is after Almassy and Dickerson (1978) and is based on X-ray crystallographic structures for tuna cytochrome *c* (Swanson et al., 1977; Takano et al., 1977; Mandel et al., 1977; Takano & Dickerson 1981a,b) and the two yeast iso cytochromes *c*, iso-1 (Louie et al., 1988) and iso-2 (G. D. Brayer, unpublished results).

is less stable than normal in vivo but which is at least partially functional (Wood et al., 1988). Folding properties of purified Thr-30 iso-2, however, are as yet unknown.

Pro-76 is the second residue of a type II reverse turn and is partially exposed on the protein surface. Replacement of Pro-76 by glycine leads to a protein less stable than the normal protein (Figure 1) but which folds to a nativelike conformation (Wood et al., 1988). The kinetic properties of folding/unfolding of the mutant and normal proteins are similar in many respects, but there are clear differences. A mutation-induced increase in rate is observed for fast unfolding (phase τ_2), and the absorbance-detected slow folding phase (τ_{1a}) is absent for the mutant protein.

Fast and Slow Phases in Fluorescence-Detected Refolding Are Unchanged. When monitored by fluorescence, refolding of Gly-76 iso-2 is indistinguishable from refolding of normal iso-2 (Figures 2, 5, and 6). Even the relative amplitudes extrapolate to similar values (0.8–0.9) in the limit of low Gdn·HCl concentration (Figure 5 vs Figure 6). Rates of fluorescence-detected refolding (phases τ_2 and τ_{1b}) are also unchanged for replacements of Pro-71 in iso-2 (White et al., 1987) and iso-1-MS cytochromes *c*[3] (Ramdas & Nall, 1986). The present results together with those obtained previously suggest that if imide isomerization is involved in fluorescence-detected slow folding, the species responsible must be unfolded polypeptide chains with Pro-25 or Pro-30 in nonnative formats (see Table II and Figure 7).

The physical processes involved in the rate-limiting step in fast folding (phase τ_{2b}) are unknown, but characterization of the effects of mutations on the rate of fast folding provides the kind of information that can help in deciding between alternative hypotheses. For example, since the rate of fast refolding is unchanged in Gly-76 iso-2, the mutation probably perturbs a stage in fast folding following the transition state

[3] Thr-71 iso-1-MS and Ile-71 iso-1-MS have an additional fast refolding phase (τ_3). For normal iso-1-MS, phase τ_3 is observed in unfolding but not refolding. The least perturbed mutant, Val-71 iso-1-MS, behaves like normal (Pro-71) iso-1-MS in that phase τ_3 is observed in unfolding but not for refolding (Ramdas & Nall, 1986).

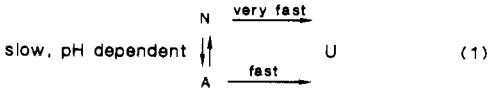
Table II: Replacements of Prolines and Slow Folding Phases[a]

protein	slow phase detected by fluorescence pH 7.2	fluorescence pH 5–6	absorbance pH 7.2	absorbance pH 5–6	ref[d]
Native Proteins					
iso-1-MS	+	+	+	+	1–3
iso-2	+	+	+[b]	+	4–7
Mutant Proteins					
Pro-30					
Thr-30 iso-2	nd	nd	nd	nd	8
Pro-71					
Val-71 iso-1-MS	nd	+	±[c]	+	2, 3, 9
Ile-71 iso-1-MS	nd	+	±[c]	+	2, 3, 9
Thr-71 iso-1-MS	nd	+	±[c]	+	2, 3, 9
Thr-71 iso-2	+	+	−	+	10
Pro-76					
Gly-76 iso-2	+	+	−	−	11

[a] A summary of normal yeast iso cytochromes *c* and mutant forms in which conserved prolines have been replaced by other amino acids. The presence (+) or absence (−) of the absorbance- and the fluorescence-detected slow phases is indicated for refolding at two final pH values. Detection of a phase with a substantially reduced amplitude is indicated (±). For the mutant proteins, the nativelike conformation is favored at pH 5–6 while pH 7.2 favors a mixed folded state containing nativelike and alkaline species. For the normal iso cytochromes *c*, only the native conformation is present between pH 5 and 8. In some cases, slow folding has not been monitored (nd). [b] Iso-2 folds to the native conformation for pH 5–7.2, and the absorbance-detected slow folding phase is observed with full amplitude. For folding to the alkaline (or mutant) conformation at high pH, the absorbance-detected slow phase disappears. At intermediate pH, the decrease in the amplitude of the absorbance-detected slow phase is correlated with a decrease in the fraction of native species relative to alkaline species in the final conditions (Nall et al., 1988). [c] Compared to folding at pH 5–6, the amplitude of the absorbance-detected slow folding phase at pH 7.2 is decreased by 50% for Val-71 iso-1-MS and Ile-71 iso-1-MS and decreased by 70% for Thr-71 iso-1-MS. The absorbance-detected phase is absent for folding of Thr-71 iso-2 at pH 7.2. The amplitude for absorbance-detected slow folding appears to be correlated with the fraction of nativelike species in the final conditions (Ramdas, 1987). [d] References are the following: (1) Zuniga & Nall, 1983; (2) Ramdas & Nall, 1986; (3) Ramdas, 1987; (4) Nall & Landers, 1981; (5) Nall, 1983; (6) Osterhout & Nall, 1985; (7) Nall et al., 1988; (8) Wood et al., 1988; (9) Ernst et al., 1985; (10) White et al., 1987; (11) this work.

(Tanford, 1968; Beasty et al., 1987).

Fast Unfolding Is Increased in Rate. Replacement of Pro-76 with glycine increases the rate of fast unfolding (phase τ_2) but also results in a new slightly slower unfolding phase (Figures 5 and 6). For normal iso-2, an additional phase in unfolding is observed too, but only at elevated pH (Table I). For both proteins, the additional phase is probably unfolding from an altered conformational state since (1) detection of the phase for both iso-2 and Gly-76 iso-2 is correlated with the presence of mutant (or alkaline) folded species in the initial conditions for unfolding experiments and (2) unfolding from a state consisting only of mutant (or alkaline) species occurs in a single (slower) phase for both iso-2 and Gly-76 iso-2 (Table I). If the new phase is unfolding from the mutant conformation, the rate of equilibration between folded nativelike and folded mutant conformations must be slow compared to the rate of either unfolding reaction. This appears to be the case (eq 1; also compare Figure 4 to Figures 5 and



(1)

6). The faster of the two unfolding phases is identified with unfolding of the nativelike species. Over a range of final Gdn·HCl concentrations, phase τ_2 for unfolding of Gly-76 iso-2

is increased in rate by a factor of 2–3 compared to normal iso-2 (Figures 5 and 6). This suggests that replacement of Pro-76 by glycine perturbs an event that precedes the transition state for fast unfolding.

Gly-76 Iso-2 Folds to a Mutant Conformation via the Nativelike State. Slow changes in absorbance in a direction opposite to that expected for folding are observed following refolding of Gly-76 iso-2 (Figure 3A,C). A likely explanation is a slow equilibration among folded species; that is, Gly-76 iso-2 folds preferentially to a nativelike conformation but is partially converted to mutant or alkaline species following refolding (eq 2). Several observations support this hypothesis.

$$U \xrightarrow{\text{fast}} N \underset{\text{pH dependent}}{\overset{\text{slow}}{\rightleftharpoons}} A \qquad (2)$$

(1) The pH dependence of the nativelike to mutant conformational equilibrium (pK = 6.71; L. Ramdas, unpublished results) indicates that the final equilibrium state at pH 6 will be composed of both nativelike (84%) and mutant (16%) species. (2) The absorbance changes are in a direction opposite to that expected for refolding but have the proper sign for net conversion of nativelike to mutant species. (3) The slow reaction that follows folding has the same rate and pH dependence as the conformational change between the nativelike and mutant folded states (Figure 4). Thus, the small amounts of mutant species present at equilibrium *do not* appear to be formed directly from the unfolded protein. Instead, folding first proceeds to the nativelike conformation and is followed by a conformational change to an altered mutant conformation. Preference for folding to the nativelike conformation prior to a slow conformational conversion to a nonnative state has also been observed for folding of alkaline iso-2 (Nall, 1986). Folding to mutant conformations by passage through a nativelike state may be a general phenomenon with important mechanistic implications. If folding pathways are highly conserved, mutations that block the normal pathway will generate proteins that never fold and cannot be isolated and studied. Isolatable mutant proteins may be required to fold to nativelike conformations along the usual pathway. Highly perturbed mutant proteins that take on nonnative conformations at equilibrium appear to follow the kinetically preferred pathway to a nativelike state prior to conformational equilibration with the altered structure.

Gly-76 Iso-2 Folds to a Nativelike State without an Absorbance-Detected Slow Phase. For Gly-76 iso-2, there is no evidence of the absorbance-detected slow phase seen in refolding of normal (Pro-76) iso-2 (Figure 3). Possible causes for loss of the slow phase are (1) folding proceeds to a mutant conformation along a new pathway that bypasses the absorbance-detected slow step and (2) the slow process is generated by imide isomerization of Pro-76, so replacement of Pro-76 by glycine allows the protein to fold along the native pathway at a faster rate. For Gly-76 iso-2, the former explanation is favored for folding to the mutant conformation above neutral pH, but the latter explanation is favored for folding to the nativelike conformation below neutral pH. Both normal iso-2 and Thr-71 iso-2 contain a proline at position 76 but lack the absorbance-detected slow phase when folding to mutant (or alkaline) conformations (Nall et al., 1988; White et al., 1987). For these proteins, and probably Gly-76 iso-2 as well, formation of the mutant (or alkaline) conformation does not require the slow absorbance-detected process. Folding of Gly-76 iso-2 to a *nativelike conformation* (Figure 3A), however, also occurs in the absence of the absorbance-detected slow phase. For all other normal or mutant proteins (Table II), folding to the nativelike conformation involves absorbance-detected slow folding, suggesting that, in general, the absorbance-detected slow phase is required for folding to a nativelike conformation. The simplest conclusion is that replacement of Pro-76 by a glycine has removed the need for the slow process in folding to the nativelike conformation; i.e., the absorbance-detected slow folding species are generated by isomerization of the Pro-76 imide bond to a nonnative format.

Conclusions. Replacement of Pro-76 by glycine in iso-2 leads to a protein with decreased stability toward Gdn·HCl-induced unfolding. Compared to normal iso-2, both fast and slow fluorescence-detected phases of refolding of Gly-76 iso-2 are unchanged. Fast unfolding of Gly-76 iso-2 from the nativelike conformation is increased in rate. A new fluorescence-detected unfolding phase has been assigned to unfolding of mutant species present in the initial conditions. A slow absorbance-detected conformational change is detected following refolding. The slow reaction involves a net conversion of nativelike to mutant species, suggesting that the preferred pathway for folding to the mutant conformation is via the nativelike conformation. Unlike the two normal iso cytochromes *c* and several proline substitution mutants, Gly-76 iso-2 folds to the nativelike conformation without the usual absorbance-detected slow phase. Apparently, the absorbance-detected slow folding species are generated by isomerization of the Pro-76 imide bond.

ACKNOWLEDGMENTS

We thank Gary Brayer, Heinrich Roder, and Yiqing Feng for providing helpful comments and permission to refer to unpublished results. Efrain Zuniga is thanked for help with yeast fermentations, protein purifications, and preparation of figures.

Registry No. Cytochrome *c*, 9007-43-6; proline, 147-85-3.

REFERENCES

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

Babul, J., Nakagawa, A., & Stellwagen, E. (1978) *J. Mol. Biol. 126*, 117.

Beasty, A. M., Hurle, M., Manz, J. T., Stackhouse, T., & Matthews, C. R. (1987) in *Protein Engineering* (Oxender, D. L., & Fox, C. F., Eds.) pp 91–102, Alan R. Liss, New York.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 953.

Brandts, J. F., Brennan, M., & Lin, L.-N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4178.

Dickerson, R. E. (1972) *Sci. Am. 226*, 58.

Ernst, J. F., Hampsey, D. M., Stewart, J. W., Rackovsky, S., Goldstein, D., & Sherman, F. (1985) *J. Biol. Chem. 260*, 13225.

Fischer, G., & Bang, H. (1985) *Biochim. Biophys. Acta 828*, 39.

Hampsey, D. M., Das, G., & Sherman, F. (1985) *J. Biol. Chem. 261*, 3259.

Kelley, R. F., & Richards, F. M. (1987) *Biochemistry 26*, 6765.

Krebs, H., Schmid, F. X., & Jaenicke, R. (1983) *J. Mol. Biol. 169*, 619.

Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature (London) 329*, 268.

Lin, L.-N., & Brandts, J. F. (1983) *Biochemistry 22*, 559.

Lin, L.-N., & Brandts, J. F. (1984) *Biochemistry 23*, 5713.

Louie, G. V., Hutcheon, W. L. B., & Brayer, G. D. (1988) *J. Mol. Biol. 199*, 295.

Mandel, N., Mandel, G., Trus, B. L., Rosenberg, J., Carlson, G., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 4619.
Nall, B. T. (1983) *Biochemistry 22*, 1423.
Nall, B. T. (1986) *Biochemistry 25*, 2974.
Nall, B. T., & Landers, T. A. (1981) *Biochemistry 20*, 5403.
Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317.
Nall, B. T., Osterhout, J. J., Jr., & Ramdas, L. (1988) *Biochemistry 27*, 7310-7314.
Osterhout, J. J., Jr., & Nall, B. T. (1985) *Biochemistry 24*, 7999.
Osterhout, J. J., Jr., Muthukrishnan, K., & Nall, B. T. (1985) *Biochemistry 24*, 6680.
Poerio, E., Parr, G. R., & Taniuchi, H. (1986) *J. Biol. Chem. 261*, 10976.
Ramdas, L. (1987) Ph.D. Thesis, University of Texas Health Science Center, Houston, TX.
Ramdas, L., & Nall, B. T. (1986) *Biochemistry 25*, 6959.
Ramdas, L., Sherman, F., & Nall, B. T. (1986) *Biochemistry 25*, 6952.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Schmid, F. X. (1982) *Eur. J. Biochem. 128*, 77.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764.
Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 133*, 285.
Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 759.
Takano, T., & Dickerson, R. E. (1981a) *J. Mol. Biol. 153*, 79.
Takano, T., & Dickerson, R. E. (1981b) *J. Mol. Biol. 153*, 95.
Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O., Swanson, R., & Dickerson, R. E. (1977) *J. Biol. Chem. 252*, 776.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Tsong, T. Y. (1974) *J. Biol. Chem. 249*, 1988.
White, T. B., Berget, P. B., & Nall, B. T. (1987) *Biochemistry 26*, 4358.
Wood, L. C., Muthukrishnan, K., White, T. B., Ramdas, L., & Nall, B. T. (1988) *Biochemistry* (preceding paper in this issue).
Zuniga, E. H., & Nall, B. T. (1983) *Biochemistry 22*, 1430.

Carbonyl ^{13}C NMR Spectrum of Basic Pancreatic Trypsin Inhibitor: Resonance Assignments by Selective Amide Hydrogen Isotope Labeling and Detection of Isotope Effects on ^{13}C Nuclear Shielding†

Erik Tüchsen* and Poul Erik Hansen

Institute of Life Sciences and Chemistry, University of Roskilde, P.O. Box 260, DK-4000 Roskilde, Denmark

Received March 23, 1988; Revised Manuscript Received August 16, 1988

ABSTRACT: The carbonyl region of the natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum of basic pancreatic trypsin inhibitor is examined, and 65 of the 66 expected signals are characterized at varying pH and temperature. Assignments are reported for over two-thirds of the signals, including those of all buried backbone amide groups with slow proton exchange and all side-chain carbonyl groups. This is the first extensively assigned carbonyl spectrum for any protein. A method for carbonyl resonance assignments utilizing amide proton exchange and isotope effects on nuclear shielding is described in detail. The assignments are made by establishing kinetic correlation between effects of amide proton exchange observed in the carbonyl ^{13}C region with development of isotope effects and in the amide proton region with disappearance of preassigned resonances. Several aspects of protein structure and dynamics in solution may be investigated by carbonyl ^{13}C NMR spectroscopy. Some effects of side-chain primary amide group hydrolysis are described. The main interest is on information about intramolecular hydrogen-bond energies and changes in the protein due to amino acid replacements by chemical modification or genetic engineering.

A variety of information about protein structure and dynamics in solution may be obtained from the carbonyl ^{13}C NMR[1] spectrum (Gurd & Rothgeb, 1979; Kainosho et al., 1985b; Henry et al., 1987a,b). Of particular interest is the information inherent in this spectral region about the intramolecular hydrogen bonds that stabilize the backbone conformation. Knowledge about the stability and energy of these hydrogen bonds in the solution structure of the protein is essential for the understanding of protein folding, conformational dynamics, and hydrogen exchange.

The interest in the carbonyl region is boosted by recent reports on the deuterium isotope effects on ^{13}C chemical shifts in model systems containing carbonyl and alcohol or amino groups (Reuben, 1986, 1987; Hansen, 1986). These model systems may be considered as remote analogues of amide groups in proteins. Large and variable deuterium isotope

† Supported by a grant from the Danish Natural Sciences Research Council.

[1] Abbreviations: NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance; BPTI, bovine pancreatic trypsin inhibitor; COSY, correlated spectroscopy; 2D, two dimensional; FID, free induction decay; TMS, tetramethylsilane; TSP, (tetramethylsilyl)propionate.

0006-2960/88/0427-8568$01.50/0