Characterization of Folding Intermediates Using Prolyl Isomerase†

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Received July 27, 1993; Revised Manuscript Received November 8, 1993®

ABSTRACT: Structure–reactivity relationships of human peptidyl prolyl *cis–trans* isomerase (PPI) toward the two slow folding reactions of yeast iso-2 cytochrome *c* have been used to characterize the structure of folding intermediates in the vicinity of critical prolines. We propose that the relative catalytic efficiency of PPI for the protein substrate relative to a peptide substrate, $(k_{cat}/K_m)_{rel}$, is a measure of structure in folding intermediates. The structural stability of slow-folding intermediates as detected by changes in $(k_{cat}/K_m)_{rel}$ was investigated using two structural perturbants: guanidine hydrochloride and site-directed mutagenesis. Neither of the two slow folding reactions for wild-type cytochrome *c* is catalyzed at low denaturant concentrations. However, both phases are catalyzed at moderate concentrations of guanidine hydrochloride. A mutation in cytochrome *c* enhances catalysis of the fluorescence-detected slow folding phase. For protein substrates destabilized by denaturants or mutation, we suggest that increases in $(k_{cat}/K_m)_{rel}$ result from a loosening of the substrate structure, providing better access of peptidyl prolyl isomerase to critical proline(s).

Many of the kinetic complexities encountered during protein folding result from fast- and slow-folding forms of the unfolded protein (Garel & Baldwin, 1973). The slow-folding species in a number of proteins are produced by proline *cis–trans* isomerization (Brandts *et al.*, 1975; Schmid & Baldwin, 1978; Kelley & Richards, 1987; Wood *et al.*, 1988a; Kiefhaber *et al.*, 1990). Recent investigations indicate that peptidyl prolyl *cis–trans* isomerase (PPI)[1] catalyzes the slow folding reactions of some proteins (Fischer & Bang, 1985; Schönbrunner *et al.*, 1991; Lang *et al.*, 1987; Bachinger, 1987; Schmid, 1993) and allows identification of kinetic phases that arise as a result of proline isomerization.

Iso-2 is a small globular protein which can be unfolded reversibly. Distinct slow folding reactions have been observed using each of two optical probes: absorbance and fluorescence (Nall & Landers, 1981; Nall, 1983). Depending on the final conditions, the fluorescence-detected phase is 3–20-fold faster in rate than the absorbance-detected phase. For refolding ending in the native baseline region, the relative amplitude associated with the absorbance-detected phase (α_1^a) is somewhat larger ($\alpha_1^a = 0.2$ –0.35) than the relative amplitude of the fluorescence-detected phase ($\alpha_1^b = 0.15$–0.2). The relative amplitudes of both slow reactions decrease for final folding/unfolding conditions ending within the equilibrium unfolding transition zone and go to zero for unfolding experiments ending above the transition zone (Nall & Landers, 1981; Nall, 1983). The reasons for coupling of the different slow folding reactions to distinct optical probes are not known but must be related to the structures of folding intermediates. Fluorescence changes can be attributed principally to the average distance between the tryptophan residue (donor) and the heme (quencher). Absorbance changes in the visible spectral region reflect heme environment and ligation state, while changes in the ultraviolet spectral region arise from changes in solvation of aromatic groups (Nall, 1983). Double-jump measurements of the rates of formation of the slow-refolding species show that the absorbance-detected slow-folding species are generated in the unfolded protein on a distinctly different time scale than the fluorescence-detected slow-folding species. Thus different slow processes in the unfolded protein generate the fluorescence-detected and absorbance-detected species (Osterhout & Nall, 1985). Experiments presented here show that under appropriate conditions both slow phases in refolding are catalyzed by the enzyme peptidyl prolyl *cis–trans* isomerase, and so both slow reactions involve prolyl *cis–trans* isomerization. Folding studies of site-directed mutant proteins have shown that the absorbance-detected slow phase is generated by isomerization of Pro76 (Wood et al., 1988a). In contrast, no single point mutation replacing any of the remaining prolines (Pro71, Pro30, Pro25, Pro-1) completely eliminates the fluorescence-detected slow phase (Ramdas & Nall, 1986; White et al., 1987; C. MacKinnon and S. Veeraraghavan, unpublished; W. A. McGee and S. D. Rodriguez, unpublished). Hence, we believe that the fluorescence-detected slow- refolding species are a mixture of species with non-native proline isomers generated by isomerization in the unfolded state of two or more of the remaining prolines: Pro71, Pro30, Pro25, or Pro-1.

Are there differences in the structures near different critical prolines in folding intermediates? Are the stabilities of these structures similar? In the present study, we have used the structure–reactivity relationship of PPI toward wild-type and mutant iso-2 to address these questions. In addition, the

† Supported by grants from the National Institute of General Medical Sciences (GM32980) and the Robert A. Welch Foundation (AQ838).

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® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

[1] Abbreviations: PPI, peptidyl prolyl *cis–trans* isomerase, also known as human cyclophilin, a cyclosporin A binding protein; iso-2, iso-2 cytochrome *c* from the yeast *Saccharomyces cerevisiae*; P25G iso-2, iso-2 in which proline 25 (mammalian cytochrome *c* numbering) is replaced by glycine; suc-AAPF-*p*-nitroanilide, tetrapeptide substrate used in the determination of the specific activity of PPI (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; k_{cat}/K_m, catalytic efficiency of PPI; $(k_{cat}/K_m)_{pep}$, catalytic efficiency of PPI toward the peptide substrate; $(k_{cat}/K_m)_{prot}$, catalytic efficiency of PPI toward protein substrate (cytochrome *c*); $(k_{cat}/K_m)_{rel}$, catalytic efficiency of PPI toward the protein substrate relative to peptide substrate; CsA, cyclosporin A.

0006-2960/94/0433-687$04.50/0

dependence of catalysis on the concentration of a structure-destabilizing agent, Gdn·HCl, has been explored. We have determined that both absorbance- and fluorescence-detected slow-folding phases of iso-2 are catalyzed by PPI and that catalysis is more efficient under conditions that diminish ordered structure in the vicinity of critical prolines. In this report, we present data that suggest the formation of structured intermediates during the slow folding reactions of yeast iso-2 cytochrome *c*.

MATERIALS AND METHODS

Iso-2 Cytochrome c. Normal and mutant iso-2 were purified according to published procedures (Nall & Landers, 1981), and purity was verified using SDS–polyacrylamide gel electrophoresis. A mutant form of the protein, P25G iso-2, was constructed by Dr. C. MacKinnon. Protein concentration was determined using the molar extinction coefficients ϵ_{410} = 106 100 M^{-1} cm^{-1} and ϵ_{434} = 22,700 M^{-1} cm^{-1} (Margoliash & Frohwirt, 1959).

Prolyl Isomerase. The pHNg/XA90 *Escherichia coli* cells that overexpress human PPI (human cyclophilin) were a generous gift of Dr. Christopher Walsh. PPI was purified in the presence of a reducing agent (Liu *et al.*, 1990) and chromatographed using CM-Sephadex cation-exchange resin, from which bound PPI was eluted using a 0 to 0.25 M sodium chloride gradient. Purified PPI was stored at 4 °C for use within two months, or at −70 °C for use within six months. Enzyme concentration was determined by a Bradford assay (Bradford, 1976) and/or by using the molar extinction coefficient ϵ_{280} = 8730 M^{-1} cm^{-1} calculated on the basis of aromatic amino acid content (Gill & Von Hippel, 1989). Specific activity of PPI, $(k_{cat}/K_m)_{pep}$, was measured according to Fischer *et al.* (1990). The effect of Gdn·HCl on the specific activity was determined by conducting the assays in the presence of appropriate concentrations of the denaturant, under the same conditions as for iso-2 refolding experiments. To minimize heme reduction by the DTT present in the PPI stock solution, PPI was subjected to gel filtration using Sephadex G-25 spinning column chromatography immediately prior to its use in refolding experiments with cytochrome *c*. While the presence of reducing agent during purification of PPI does not affect catalysis of the fluorescence-detected slow folding reaction of iso-2, PPI purified in the absence of reducing agent fails to catalyze the absorbance-detected slow folding phase.

Cyclosporin A used for inhibition of PPI activity was a generous gift of Sandoz Pharmaceuticals Corporation. Inhibition of PPI activity was achieved by incubating PPI (10 μM) with CsA (50–100 μM) at 4 °C until the specific activity of the enzyme was about 1% of the initial value. The sample was filtered to remove precipitated CsA by passage through a 0.2 μm Acrodisc 13 (Gelman Scientific). The concentration of the CsA-inhibited PPI was determined spectrophotometrically prior to use in refolding experiments.

Fluorescence-Detected Slow Folding. Oxidized iso-2 was used throughout the study. All folding experiments were conducted in 0.1 M sodium phosphate at pH 6.0, 20 °C, and were initiated by Gdn·HCl dilution. Catalyzed refolding experiments were performed by manual mixing of unfolded iso-2 stock (in 3 or 3.9 M Gdn·HCl) with the dilution buffer containing PPI. Preliminary studies of fluorescence-detected slow folding at 5 μM iso-2 were carried out using an SLM-Aminco SPF-500C spectrofluorimeter (SLM Instruments Inc.), while kinetic measurements at high PPI concentrations were performed at 20 μM iso-2 using a Bio Logic SFM-3 stopped-flow spectrofluorimeter (Molecular Kinetics, Pullman, WA). The stopped-flow mixing apparatus was mounted so that the solutions flowed horizontally rather than in the usual vertical mode. The stock solution of iso-2 containing a high concentration of Gdn·HCl was placed in syringe 1 (lowest syringe). Phosphate buffer and PPI solution were placed in syringes 2 and 3, respectively. This arrangement minimized premature mixing due to density differences between the enzyme and Gdn·HCl stocks. For stopped-flow experiments at 5 μM PPI, the iso-2 stock was diluted in two stages: 1:10 with buffer in the first mixer, followed by 1:2 with PPI in the second mixer to attain a total dilution of 20-fold. Experiments that measured $(k_{cat}/K_m)_{prot}$ for the fluorescence-detected phase were carried out by varying the PPI concentrations (0–15 μM) using variable-ratio mixing. Fluorescence changes that accompany initiation of refolding were monitored at 350 nm with excitation at 285 nm.

Absorbance-Detected Slow Folding. Absorbance-detected slow folding reactions of iso-2 were initiated by manual mixing using a 1-cm-path-length cuvette and monitored at 394 nm using an HP8452A UV-visible spectrophotometer. A final concentration of 20 μM iso-2 was used. All other experimental conditions were as described above. In some cases stopped-flow mixing was used to monitor absorbance-detected slow folding. These experiments were carried out in a manner similar to that described for the fluorescence-detected stopped-flow experiments, except that folding was followed by absorbance changes at 394 nm.

Data Reduction. Kinetic data from the absorbance-detected and fluorescence-detected folding reactions were converted to the Bio-Kine data file format using a data file format conversion program and analyzed using the Bio-Kine software (Molecular Kinetics, Inc., Pullman, WA). Apparent first-order rate constants (k_{obs}) were obtained by nonlinear least squares fits of the data according to the equation

$$A_\infty - A(t) = [A_\infty - A_o] \exp(-k_{obs}t) \quad (1)$$

where A_o is absorbance or fluorescence at $t = 0$, A_∞ the final absorbance or fluorescence, and $A(t)$ is the measurement at time t.

The specific activity, $(k_{cat}/K_m)_{pep}$, for the peptide substrate, suc-AAPF-*p*-nitroanilide, was determined according to Harrison and Stein (1990) using the equation

$$(k_{cat}/K_m)_{pep} = (k_{obs} - k_u)/[PPI] \quad (2)$$

where k_{obs} is the observed first-order rate constant (at 390 nm); k_u is the uncatalyzed rate, and [PPI] is the concentration of PPI used in the assay. The catalytic efficiency for the protein substrate, $(k_{cat}/K_m)_{prot}$, was determined in the same way using eq 2 or from the slope of a plot of k_{obs} vs [PPI] (Figure 2). The relative catalytic specificity, $(k_{cat}/K_m)_{rel}$ is given by

$$(k_{cat}/K_m)_{rel} = (k_{cat} - k_m)_{prot}/(k_{cat}/K_m)_{pep} \quad (3)$$

which normalizes the observed $(k_{cat}/K_m)_{prot}$ values and corrects for loss of enzyme activity in the presence of Gdn·HCl.

Gdn·HCl Dependence of PPI Specific Activity. The specific activity, $(k_{cat}/K_m)_{pep}$, of the enzyme was determined using the peptide substrate suc-AAPF-*p*-nitroanilide at 0.1 M sodium phosphate, pH 6, and 20 °C. Assays carried out with 20 nM PPI show that the value obtained for $(k_{cat}/K_m)_{pep}$ is independent of peptide concentration between 5 and 360 μM peptide. Between 0 and 0.7 M Gdn·HCl, the $(k_{cat}/K_m)_{pep}$ decreases according to the equation $(k_{cat}/K_m)_{pep} = \{1.0 - (2.2\ M^{-1})[\text{Gdn·HCl}] + (1.6\ M^{-2})[\text{Gdn·HCl}]^2\}(k_{cat}/K_m)^\circ_{pep}$, with denaturant concentration in units of M and where $(k_{cat}/K_m)^\circ_{pep}$

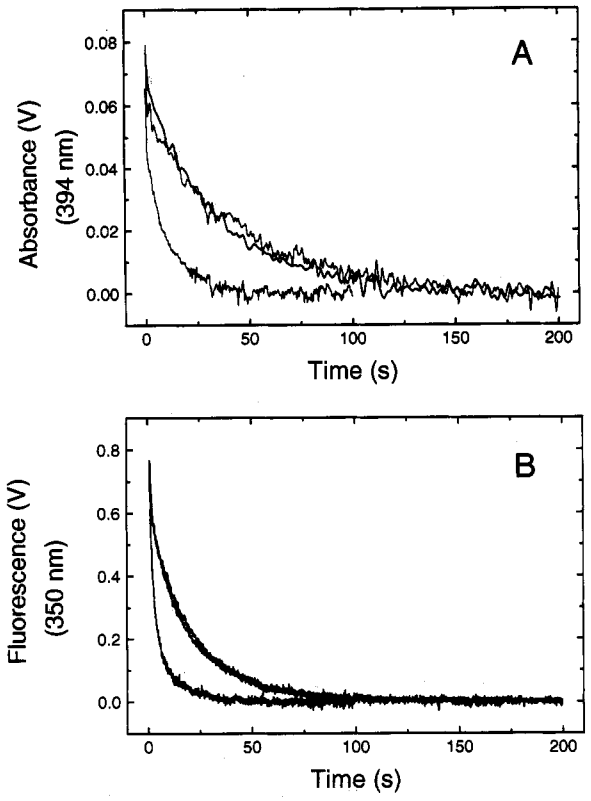

FIGURE 1: Absorbance-detected (A) and fluorescence-detected (B) slow folding kinetics of wild-type iso-2. Experiments were carried out in the presence or absence of peptidyl prolyl isomerase (PPI) and cyclosporin A, an inhibitor of PPI. For absorbance-detected refolding (A) the upper trace is for refolding in the presence of 5 μM PPI inhibited with cyclosporin A, the middle trace is for refolding in the absence of PPI, and the lower trace is for refolding in the presence of 5 μM PPI. For fluorescence-detected refolding (B) the upper trace is for refolding in the presence of 5 μM PPI inhibited by cyclosporin A, the middle trace is for refolding in the absence of PPI, and the lower trace is for refolding in the presence of 5 μM PPI. Refolding was induced by diluting iso-2 unfolded in 3.0 M Gdn·HCl with sodium phosphate buffer to give final concentrations of 0.6 M Gdn·HCl and 18 μM iso-2 for fluorescence or 39 μM iso-2 for absorbance. Other conditions were 0.1 M sodium phosphate, pH 6.0, and 20 °C.

is the value for purified enzyme in the absence of Gdn·HCl. Generally, $(k_{cat}/K_m)°_{pep}$ for a freshly purified enzyme preparation is 2×10^7 M^{-1} s^{-1}. Within 4 weeks of storage at 4 °C, this drops to 1×10^7 M^{-1} s^{-1} and remains unchanged thereafter for at least 5 more weeks.

The sensitivity of the activity assay depends on a variety of factors, one being the difference between the uncatalyzed rate (k_u) and the catalyzed rate (k_{obs}). Within the limitations of enzyme and substrate solubility and the time resolution of the kinetic instrumentation, the difference $k_{obs} - k_u$, can be increased by increasing the PPI concentration (see eq 2). In practice this has allowed the sensitivity of the assay to be maintained within about ± 0.1 (k_{cat}/K_m) over the 0–0.7 M Gdn·HCl concentration range for both peptide and protein substrates.

RESULTS

PPI Catalysis of Slow Refolding Reactions of Cytochrome c. Changes in the Soret region (394 nm) of the absorbance spectrum that accompany the slow refolding of wild-type iso-2 cytochrome c indicate that the absorbance-detected slow folding reaction is a kinetic phase with a rate of about 0.03 s^{-1} (Figure 1A). For refolding under the same final conditions the fluorescence-detected slow phase is slightly faster with a rate of 0.06 s^{-1} (Figure 1B). Both the absorbance-detected and the fluorescence-detected slow folding reactions are catalyzed by PPI, but in the presence of saturating amounts of the PPI inhibitor cyclosporin A, neither phase is catalyzed (Figure 1A,B). This indicates that catalysis of folding is a consequence of the peptidyl prolyl isomerase activity of PPI. Rates for the absorbance-detected (data not shown) and fluorescence-detected (Figure 2) slow folding reactions of normal iso-2 increase linearly with increasing PPI concentrations. The rate of the corresponding fluorescence-detected fast folding reaction is the same in the presence and absence of PPI, suggesting that catalysis by PPI is limited to the slow kinetic phases in folding (data not shown). This is as expected if only the slow folding phases involve proline imide bond isomerization.

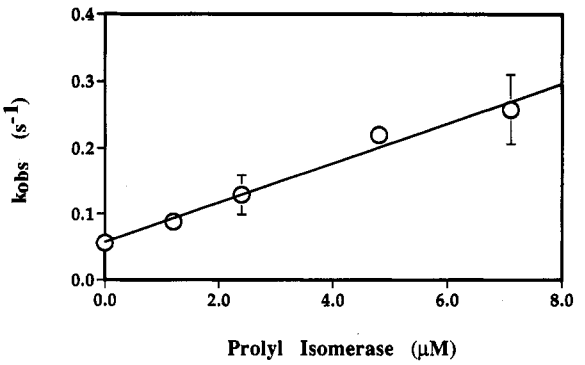

FIGURE 2: Determination of the catalytic efficiency, $(k_{cat}/K_m)_{prot}$, for the fluorescence-detected slow folding reaction of wild-type iso-2. The protein substrate was refolded at a final concentration of 0.6 M Gdn·HCl in the presence of varying amounts of PPI. The solid line through the data points is a least squares fit to the equation $k_{obs} = (k_{cat}/K_m)_{prot}[PPI] + k_u$, where $(k_{cat}/K_m)_{prot} = 2.99 \times 10^4$ M^{-1} s^{-1} and $k_u = 0.0575$ s^{-1}. Conditions for refolding cytochrome c were as described in Figure 1. Each data point is the average of at least three determinations. Error bars are included when the standard deviation exceeds the size of the symbols.

Guanidine Dependence of PPI-Catalyzed Slow Folding Reactions of Iso-2 Cytochrome c. The $(k_{cat}/K_m)_{rel}$ of PPI for the fluorescence-detected slow phase of wild-type iso-2 increases with increasing Gdn·HCl concentration (Figure 3). The sensitivity of our assay makes it impossible to measure PPI catalysis of this reaction below 0.1 M denaturant. The $(k_{cat}/K_m)_{rel}$ for the absorbance-detected slow folding phase of iso-2 also increases with an increase in denaturant concentration (Figure 3). Here, the assay sensitivity makes it impossible to detect catalysis below ~0.23 M denaturant. Catalysis of the fluorescence-detected slow phase is somewhat better than the absorbance-detected phase as judged by the fact that the fluorescence-detected slow folding phase has a higher value of $(k_{cat}/K_m)_{rel}$ over the entire range of Gdn·HCl concentrations for which PPI catalysis of folding has been measured (Figure 3).

Gdn·HCl Dependence of PPI-Catalyzed Slow Folding of P25G Iso-2. PPI catalyzes both the absorbance-detected and the fluorescence-detected slow phases of P25G iso-2 (Figure 4). For the fluorescence-detected slow folding reaction, $(k_{cat}/K_m)_{rel}$ is greater for P25G iso-2 than for normal iso-2 at low Gdn·HCl concentrations (y-intercept) and the Gdn·HCl sensitivity (slope) is about 2-fold lower for the mutant protein compared to the wild-type protein (8.92 vs 20.3, respectively). These differences suggest that the P25G mutation induces structural changes within a folding intermediate in the vicinity of the critical prolines responsible for this phase, and that the local structural perturbations can be assessed by the reactivity of PPI toward the modified substrates.

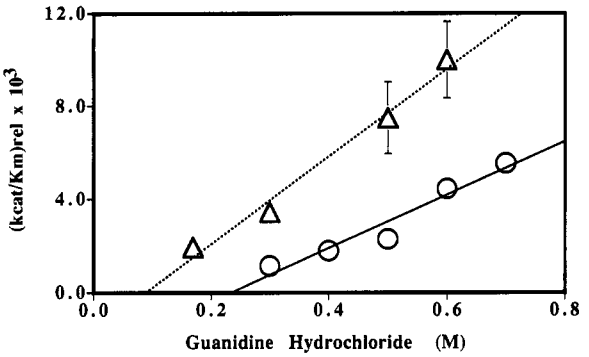

FIGURE 3: Guanidine sensitivity of PPI-catalyzed iso-2 cytochrome *c* refolding. The relative catalytic efficiency, $(k_{cat}/K_m)_{rel}$, of PPI for the fluorescence-detected (triangles, dotted line) and absorbance-detected (circles, solid line) slow folding reactions is shown as a function of the final concentration of Gdn·HCl. Refolding was initiated by dilution of a solution of cytochrome *c* unfolded in 3 or 3.9 M Gdn·HCl with 0.1 M sodium phosphate buffer to obtain the indicated final Gdn·HCl concentration. The initial cytochrome *c* concentration was varied in order to maintain a final concentration of cytochrome *c* of 20 μM. Catalyzed and uncatalyzed rates were obtained by including or excluding PPI in the mixing buffer. The value of $(k_{cat}/K_m)_{rel}$ for each concentration of Gdn·HCl was calculated as described in Materials and Methods. Other conditions were 0.1 M sodium phosphate, pH 6.0, and 20 °C. The dotted line is a least squares fit to the fluorescence-detected data according to the equation $(k_{cat}/K_m)_{rel} = (18.89[\text{Gdn·HCl}] - 1.69) \times 10^{-3}$. The solid line is a least squares fit to the absorbance-detected data according to the equation $(k_{cat}/K_m)_{rel} = (11.44[\text{Gdn·HCl}] - 2.67) \times 10^{-3}$. The lines have no theoretical significance and are included only to show that $(k_{cat}/K_m)_{rel}$ exhibits a different dependence on guanidine hydrochloride concentration for the fluorescence-detected and absorbance-detected slow folding phases. Data points are an average of three or more determinations. Error bars are included when the standard deviation exceeds the size of the symbols.

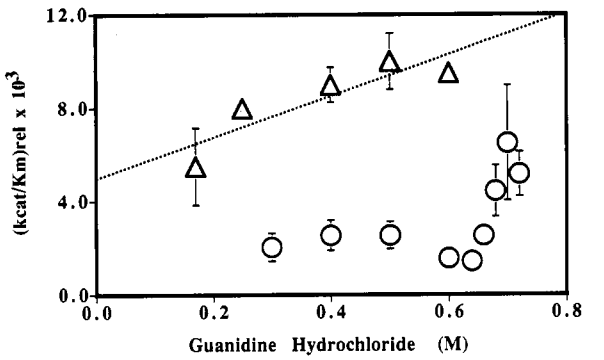

FIGURE 4: Gdn·HCl dependence of catalysis of P25G iso-2 refolding. The relative catalytic efficiency, $(k_{cat}/K_m)_{rel}$, of PPI for the fluorescence-detected (triangles, dotted line) and absorbance-detected (circles) slow folding reactions is shown as a function of the final concentration of Gdn·HCl. Experiments were carried out as described in Figure 3. Data points are an average of three or more determinations. Error bars are included when the standard deviation exceeds the size of the symbols. The dotted line through the experimental points is a least squares fit of the data for the fluorescence-detected phase according to the equation $(k_{cat}/K_m)_{rel} = (8.92[\text{Gdn·HCl}] + 4.97) \times 10^{-3}$. The line has no theoretical significance and is included only to show that $(k_{cat}/K_m)_{rel}$ exhibits a different dependence on guanidine hydrochloride concentration for the fluorescence-detected phase of P25G iso-2 compared to the fluorescence-detected slow folding phase for normal iso-2 (Figure 3). Within experimental errors, and limitations due to the onset of the equilibrium unfolding transition near 0.6 M Gdn·HCl, the dependence of $(k_{cat}/K_m)_{rel}$ on Gdn·HCl concentration for the absorbance-detected slow folding reaction is the same for P25G iso-2 and normal iso-2 (Figure 3).

For the absorbance-detected phase in folding of P25G iso-2 (Figure 4), catalysis is, within errors, the same as for the wild-type protein at low Gdn·HCl concentrations. At high denaturant concentrations the Gdn·HCl dependence of catalysis of P25G iso-2 refolding is complex. We believe that the unusual denaturant dependence of catalyzed refolding of P25G iso-2 at high Gdn·HCl concentrations results from P25G iso-2 having entered the Gdn·HCl-induced equilibrium unfolding transition. The unfolding transition for P25G iso-2 begins at ~0.6 M Gdn·HCl (data not shown), and the apparent enhancement in catalysis of folding by PPI at and above this denaturant concentration cannot be interpreted.

DISCUSSION

Slow Refolding Phases of Iso-2 and Catalysis by Prolyl Isomerase. PPI catalyzes two slow folding phases of yeast iso-2 cytochrome *c*. The results for the absorbance-detected slow folding reaction of iso-2 are similar to those with horse heart cytochrome *c* (Lin *et al.*, 1988). We have shown that catalysis of both slow phases is eliminated in the presence of cyclosporin A (Figure 1). Hence, the results described herein are a direct consequence of the *cis–trans* isomerase activity of PPI and not a chaperonin-like activity (Freskgård *et al.*, 1992).

Structure in Folding Intermediates Modulates PPI Catalysis of Slow Folding. The differences in $(k_{cat}/K_m)_{rel}$ for the two slow phases are, in principal, due to differences in sequence or structure near critical prolines. The $(k_{cat}/K_m)_{pep}$ of PPI toward peptide substrates is largely independent of the amino acid preceding a proline (Harrison & Stein, 1990). If $(k_{cat}/K_m)_{rel}$ is insensitive to protein sequence for folding intermediates too, then differences in the relative catalytic efficiency may reflect differences in the structure of folding intermediates. Presumably the structural differences would modulate the access of PPI to critical proline residues within the folding intermediates. The observed increases in $(k_{cat}/K_m)_{rel}$ on destabilizing the substrate by mutation or denaturant support this interpretation (Figures 3 and 4). Regardless, the assumption that PPI catalysis is independent of amino acid sequence requires further verification for both peptide substrates and folding intermediates.

Denaturant Enhances Catalysis of Slow Folding Reactions of Iso-2. The PPI-catalyzed fluorescence-detected and absorbance-detected slow folding reactions of iso-2 are both dependent on Gdn·HCl concentration (Figure 3). For wild-type cytochrome *c*, the $(k_{cat}/K_m)_{rel}$ for the fluorescence-detected phase extrapolates to 0 at 0.1 M Gdn·HCl, and it extrapolates to 0 at 0.23 M Gdn·HCl for the absorbance-detected phase. A linear extrapolation of $(k_{cat}/K_m)_{rel}$ must be regarded as speculative. However, one interpretation is that these Gdn·HCl concentrations define approximate lower limits of denaturant necessary for PPI accessibility to critical prolines, the isomerization of which results in the fluorescence-detected and absorbance-detected slow-folding species. Hence, the folding intermediates which accumulate behind the slow steps in folding are likely to contain compact structural elements near the critical prolines. This model is consistent with the observation that $(k_{cat}/K_m)_{rel}$ increases with increasing Gdn·HCl concentration, presumably due to Gdn·HCl-induced melting of structure which leads to improved accessibility to PPI. Gdn·HCl sensitivity is higher for the fluorescence-detected slow phase than for the absorbance-detected phase. We believe this reflects the greater lability of the structure in the neighborhood of the critical prolines, resulting in the fluorescence-detected slow-folding species, compared to the structure near Pro76 (absorbance-detected slow phase). We conclude that the structure in folding intermediates near the critical prolines that gives rise to the fluorescence-detected slow-folding species is distinctly different from the structure

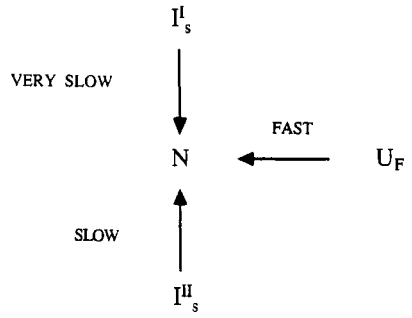

FIGURE 5: Refolding scheme for iso-2 cytochrome *c*. The scheme [see Osterhout and Nall (1985)] assumes that unfolded cytochrome *c* is a mixture of species: U_f species have both of the critical prolines in the native (*trans*) format; U^I_s species have a *cis* Pro76, but other critical prolines are *trans*; U^{II}_s species have a *trans* Pro76, but one or more other critical prolines are *cis*. When refolding is initiated, the U_f species refold rapidly in the fast kinetic phase of folding. The U^I_s species and the U^{II}_s species collapse to structured intermediate species, I^I_s and I^{II}_s, respectively. The I^I_s species have the fluorescence spectrum of the native species but an intermediate absorbance spectrum. The I^{II}_s species have the absorbance spectrum of the native species but an intermediate fluorescence spectrum. Isomerization of the *cis* proline (Pro76) to the *trans* configuration within the I^I_s species gives rise to the slow absorbance-detected phase, and isomerization of a critical *cis* proline to the *trans* configuration within the I^{II}_s species gives rise to the slow fluorescence-detected phases. PPI, a peptidyl prolyl *cis–trans* isomerase, catalyzes both slow phases in folding, but has no effect on the rate of the fast folding phase.

near the critical proline (Pro76) that gives rise to the absorbance-detected show phase, at least with regard to denaturant sensitivity.

Mutation of the Protein Substrate and PPI-Catalyzed Slow Folding of Iso-2. At Gdn·HCl concentrations below 0.4 M, catalysis of the fluorescence-detected slow phase of P25G iso-2 results in a higher $(k_{cat}/K_m)_{rel}$ than that of wild-type iso-2 (Figure 4). This probably reflects improved PPI accessibility to the prolines within the fluorescence-detected slow-folding species as a result of mutation at position 25. Increasing Gdn·HCl concentration enhances the catalysis of the fluorescence-detected slow phase of P25G iso-2 but not by as much as for the wild-type protein. Taken together, this suggests that a relatively ordered structure may exist near the critical prolines responsible for the fluorescence-detected slow folding phase and that the structure shields the prolines from PPI at low denaturant concentrations. The P25G mutation disrupts or destabilizes this structure. For P25G iso-2, measurements of the Gdn·HCl concentration dependence of k_{cat}/K_m cannot be interpreted above 0.6 M Gdn·HCl where the equilibrium unfolding transition begins. Considering only data collected at low denaturant concentration, there are no significant differences in the Gdn·HCl dependence of the absorbance-detected slow phase for P25G iso-2 compared to wild-type iso-2 (Figures 3 and 4). This suggests that there is little or no effect of the mutation at position 25 in the vicinity of Pro76, the critical proline responsible for the absorbance-detected slow phase.

Mechanism of Folding. We propose a mechanism for the slow folding reactions of iso-2 (Figure 5) similar to that presented by Osterhout and Nall (1985). A specific form of this model which we favor assumes that the critical prolines, those that generate the two slow phases catalyzed by PPI, are Pro76 for the absorbance phase (Wood et al., 1988a) and a combination of two or more of Pro71, Pro30, Pro25, or Pro-1 for the fluorescence-detected phase. The U_f species make up the largest part (73%) of the unfolded polypeptide chains and have all critical prolines in the native (*trans*) isomeric format. When refolding is initiated, these chains refold in a fast folding phase that is complete before a significant amount of slow folding has occurred. After completion of the fast phase all remaining unfolded species have critical prolines in a non-native format. These include fluorescence-detected species with at least one critical proline *cis* but a *trans* Pro76 (~8%; U^{II}_S) or the absorbance-detected species with a *cis* Pro76 but the other critical prolines *trans* (~19%; U^I_S). In principle, there should also be slow-folding species which contain *cis* critical prolines in addition to a *cis* Pro76, but these species will have a low concentration (~2%).

When refolding is initiated, the U^I_S and U^{II}_S species rapidly take on structure and collapse to the I^I_S (*cis* Pro76) and I^{II}_S intermediate species, respectively. It is the structure in these intermediate species that inhibits access of PPI to the critical prolines. Presumably, it is the structure within the I^I_S or I^{II}_S intermediate that is partially disrupted by mutation or protein denaturants, allowing improved access of PPI to the critical prolines. For catalyzed slow folding, this results in an increase in $(k_{cat}/K_m)_{rel}$ of PPI for the slow-folding intermediates. With or without catalysis the prolines in the I^I_S or I^{II}_S species isomerize to a native format (*trans* isomers), and the intermediates are converted to the native protein.

Structure within Folding Intermediates Affects the Catalytic Efficiency of PPI. If the above model is correct, several questions arise. Is the structure within the I^I_S species (*cis* Pro76) similar to the structure within the I^{II}_S species? What is the extent of the structure? Is the structure global, involving all parts of the polypeptide, or localized to a specific region? If local structure is involved, what parts of the polypeptide chain are contained within the local structure? Are similar structures formed transiently during the fast folding reactions? Information providing at least partial answers to these questions can be obtained from investigations of the effects of mutations on $(k_{cat}/K_m)_{rel}$. Locations within the protein substrate which when mutated enhance or diminish $(k_{cat}/K_m)_{rel}$ are probably part of the structure. Locations at which mutations have no effect are presumably outside the structured region. In this manner, mutations can be used to define the extent of the structured regions, whether global or localized, within the I^I_S and I^{II}_S species.

By this criterion, position 25 is clearly part of the structured region of the I^{II}_S species since a P25G substitution dramatically alters the denaturant dependence of $(k_{cat}/K_m)_{rel}$ for the fluorescence-detected slow folding phase. It is less certain whether position 25 is part of the structure of the I^I_S species since for refolding at denaturant concentrations ending below the unfolding transition zone (<~0.6 M Gdn·HCl) the denaturant dependence of $(k_{cat}/K_m)_{rel}$ for the absorbance-detected slow phase is indistinguishable for iso-2 and P25G iso-2 (Figures 3 and 4). Information about the stability of the structure within the I^{II}_S vs the I^I_S species is available from the denaturant concentration dependence of $(k_{cat}/K_m)_{rel}$. The fact that the denaturant dependence of $(k_{cat}/K_m)_{rel}$ differs for the absorbance phase compared to the fluorescence phase shows that the structure of the I^I_S species differs from that of the I^{II}_S species, at least in stability toward denaturants.

CONCLUSIONS

This study has shown how structure–reactivity studies of PPI with protein substrates can be used to characterize structure present in folding intermediates. Peptidyl prolyl isomerase (cyclophilin) catalyzes the fluorescence-detected and absorbance-detected slow folding phases of yeast iso-2 cytochrome *c*, demonstrating the involvement of prolines in both phases. The differences in $(k_{cat}/K_m)_{rel}$ between the

absorbance-detected and fluorescence-detected slow folding reactions of iso-2 show that catalysis of the absorbance-detected slow folding phase is less efficient. We interpret low values of $(k_{cat}/K_m)_{rel}$ for protein substrates to mean that structure near a critical proline blocks access of PPI to the proline. Thus the structure near Pro76 (absorbance-detected phase) is more effective in blocking access to PPI than the structure near the critical prolines that give rise to the fluorescence-detected phase.

In light of the results presented here, it seems likely that PPI catalysis of proline isomerization reactions in protein substrates may be observed more readily under slightly denaturing conditions (for the substrate macromolecule). One must, however, take precautions to avoid conditions that lead to a loss of the PPI enzymatic activity during refolding and, when necessary, correct for the partial loss of PPI specific activity. Only then can comparisons of structure and stability near critical prolines be made. Determination of the structure–reactivity relationships of PPI for various mutant protein substrates can be used to locate structured regions within folding intermediates. In addition, such studies may be useful in obtaining a better understanding of possible *in vivo* functions of prolyl isomerases.

ACKNOWLEDGMENT

We are grateful to Drs. M. H. Schreier and F. Meyer of the Pharma Division of Sandoz Ltd. for providing purified cyclophilin (PPI) which made it possible to carry out preliminary studies of PPI catalysis of iso-2 refolding. Drs. Jun Liu, C. T. Walsh, and G. L. Verdine are thanked for providing strains used to overexpress human PPI (cyclophilin) in *E. coli*. Dr. H. P. Bächinger is thanked for providing several suggestions on the purification of PPI. We are grateful to the Sandoz Research Institute of Sandoz Pharmaceuticals Corporation for providing cyclosporin A and to Dr. C. MacKinnon (Incarnate Word College, San Antonio) for constructing the P25G iso-2 mutant.

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