Development of Enzymatic Activity during Protein Folding

DETECTION OF A SPECTROSCOPICALLY SILENT NATIVE-LIKE INTERMEDIATE OF MUSCLE ACYLPHOSPHATASE*

(Received for publication, December 4, 1998, and in revised form, April 26, 1999)

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The recovery of enzymatic activity during the folding of muscle acylphosphatase and two single residue mutants (proline 54 to alanine and proline 71 to alanine) from 7 M urea has been monitored and compared with the development of intrinsic fluorescence emission. Fluorescence measurements reveal the presence in the wild-type protein of a major rapid refolding phase followed by a second low amplitude slow phase. The slow phase is absent in the fluorescence trace acquired with the proline 54 to alanine mutant, suggesting the involvement of this proline residue in the fluorescence-detected slow phase of the wild-type protein. The major kinetic phase is associated with a considerable recovery of enzymatic activity, indicating that a large fraction of molecules refolds with effective two-state behavior. The use of time-resolved enzymatic activity as a probe to follow the folding process reveals, however, the presence of another exponential slow phase arising from proline 71. This slow phase is not observable by utilizing optical probes, indicating that, unlike proline 54, the cis to trans isomerization of proline 71 can take place in an intermediate possessing a native-like fold. We suggest that, although spectroscopically silent and structurally insignificant, the cis-trans interconversion of proline residues in native-like intermediates may be crucial for the generation of enzymatic activity of functional enzymes.

The conversion of a polypeptide chain, newly synthesized from the information contained within the cellular DNA, into a native and functional protein is a key aspect of the conversion of genetic information into biological activity. This process, called protein folding, is achieved with very great efficiency compared with a random search process. Moreover, it is now well accepted that small proteins can fold in vitro without the assistance of any other protein, indicating that the information for the refolding process is contained in the sequence, and the question of how this is encoded is one of the most intriguing as well as difficult problems in structural biology.

A detailed study of the mechanism of protein folding is experimentally challenging because of the very large number of atoms and interactions present in the native state of a protein. Modern views of protein folding have pointed to the existence of an enormous number of conformations through which the polypeptide chains can in principle pass to attain the native state. The sequence of conformations traversed in the search for the native state may vary substantially between different protein molecules, a consideration that has shifted the attention of theoreticians and experimentalists from the concept of a single pathway to that of a multiplicity of pathways or trajectories in protein folding reactions (1, 2). Due to the complexity of the problem, an understanding of protein folding and the description of the events that occur upon passing from the unfolded to the native state requires a combination of experimental techniques. One approach that has proved to be extremely valuable involves the utilization of protein engineering methods to probe the contributions to efficient folding of individual residues within the protein. This can provide a detailed description of the likely structures of intermediates and transition states involved in the folding process (3, 4). Other important contributions to our understanding of protein folding mechanisms have been provided by biophysical studies aimed at monitoring the folding process in real time by means of a series of probes, such as intrinsic fluorescence, far- and near-UV circular dichroism, NMR spectroscopy, and ligand binding, each of which can monitor specifically the acquisition of native-like structure as it develops (5).

In this work, we demonstrate that it is possible to monitor the folding process of human muscle acylphosphatase (AcP)6 by means of time-resolved measurements of enzymatic activity. AcP is a 98-residue protein characterized by two antiparallel β-helices packed against a five-stranded antiparallel β-sheet (Fig. 1) (6–8). It contains two proline residues at positions 54 and 71, both in a trans configuration in the native state (Fig. 1). No disulfide bridges or cofactors are present. The protein used in the present work is a mutated form of AcP where the cysteine residue at position 21 has been replaced with a serine residue to avoid the complexities originating from a free sulfydryl group (9). The enzymatic activity of AcP is associated with the hydrolysis of acylphosphates. Substrates of this enzyme are...
species formed during the normal activity of cells, such as 1,3-bisphosphoglycerate, carbamoyl phosphate, succinyl phosphate, and the β-aspartyl phosphate associated with membrane pumps as well as synthetic acylphosphates such as acetyl phosphate and benzoylphosphate (10).

AcP has been shown to fold in an apparent two-state fashion without the accumulation of intermediates (9). A low amplitude slow phase of folding, detected with optical probes and NMR, in concurrence with the main exponential phase, has been attributed to a small fraction of molecules where cis to trans isomerization of a peptidylprolyl bond has to take place in the denatured state prior to the development of the native fold (9). However, which proline residue is involved in such a step is not yet clear. By combining intrinsic fluorescence emission and enzymatic activity as probes to follow the folding of wild-type AcP and two proline mutants (P54A and P71A), we have been able to establish a detailed folding scheme for AcP and detect an additional slow proline isomerization step that has not been observed using conventional techniques. The nature and characteristics of this step give new insight into the significance of proline cis-trans isomerization in mechanisms of protein folding.

**EXPERIMENTAL PROCEDURES**

**Materials**—The protein used in the present work is the C21S mutant of AcP. Site-directed mutagenesis was performed as described by Taddei et al. (11). AcP expression and purification was performed according to Modesti et al. (12). Protein concentration was measured by UV absorption using an ε_{280} value of 1.42 mmol⁻¹ cm⁻¹ for wild type and mutants of AcP. Benzoylphosphate was synthesized and purified as described by Camici et al. (13). All other reagents were of the highest analytical grade.

**Equilibrium Urea-induced Denaturation**—The equilibrium urea-induced unfolding of wild-type and mutated AcP was studied at 28 °C using intrinsic fluorescence emission as a probe. A Perkin-Elmer LS 50 B spectrophotometer was used with excitation and emission wavelengths of 280 and 335 nm, respectively. Each titration curve was obtained by determining the fluorescence of 21 equilibrated samples containing 0.04 mg/ml AcP in 50 mM acetate buffer, pH 5.5, and various urea concentrations at a temperature that was fitted to the equation,

\[
y_x + m_x[\text{urea}] + m_x[\text{urea}]/[1 + \exp(-\Delta G(\text{H}_2\text{O})/RT)]
\]

where \(y_x\), \(m_x\), \(y_x\), and \(m_x\) are the intercepts and slopes of the pre- and post-transition base lines, \(\Delta G(\text{H}_2\text{O})\) is the free-energy change of unfolding in the absence of denaturant, and \(m\) is the dependence of 4G on urea concentration (14). The half-denaturation urea concentration, \(C_m\), was determined by dividing \(\Delta G(\text{H}_2\text{O})\) by the \(m\) value obtained from the fitting.

**1H NMR Spectroscopy**—One-dimensional \(^1\)H NMR spectra were recorded at 25 °C in 50 mM acetate-d₄ buffer, 10% D₂O, pH 3.8, using a home-built spectrometer operating at 500.0 MHz and were processed using FELIX 2.3 (Hare Research). The water resonance was presaturated by selective irradiation during the 1.5-s relaxation delay. 1024 scans were taken using a spectral width of 6066.7 Hz. \(^1\)H chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

**Stopped-flow Fluorescence**—Folding of wild-type AcP and the mutant proteins was studied using an Applied Photophysics SX.17MV stopped-flow spectrofluorimeter. Folding was initiated by mixing the protein denatured in 7 M urea with a refolding solution. The final conditions after mixing were as follows: 0.63 M urea, 2.27% hexafluoroisopropanol alcohol (3.63% for P54A mutants), 50 mM acetate buffer, pH 5.5, 28 °C. Small amounts of hexafluorosilane alcohol were added to accelerate the fast phase of folding in order to separate it from the slower phase and allow its clear observation (9). The folding reaction was studied by monitoring the intrinsic fluorescence change above 320 nm (excitation wavelength, 280 nm) and by fitting the kinetic traces to single or double exponential functions for the determination of the relevant kinetic parameters. No significant differences in the parameters derived from the best fits (rate constants and relative amplitudes) were found when the folding process was monitored using different excitation wavelengths such as 225, 280, and 295 nm.

**Folding Followed by Time-resolved Enzymatic Activity Measurements**—The Applied Photophysics SX.17MV stopped-flow device was also used to study the recovery of enzymatic activity during the refolding of wild-type and P54A AcP. Because of its lack of enzymatic activity, this analysis could not be performed on the P71A mutant of AcP. AcP denatured in 7 M urea was mixed with a refolding solution containing benzoylphosphate as a substrate. The final conditions for individual experiments are reported in the figure legends. In all cases, the decay of 283-nm absorbance, derived from the AcP-catalyzed hydrolysis of benzoylphosphate (15), was followed after mixing the protein with the substrate.

From the time course of the absorbance, the change of enzymatic activity during the folding reaction can be obtained as follows. The rate for the AcP-catalyzed hydrolysis of substrate is given by the equation,

\[
v = 6 \times 10^4 \frac{\text{d}[\text{S}]_0}{\text{d}t}
\]

where \(v\) is the hydrolysis rate in IU/ml, \([\text{S}]_0\) is the substrate concentration, and \(6 \times 10^4\) is a factor to convert the hydrolysis rate from M/s into IU/ml. We define the relative absorbance, \(A_{rel}\), as the ratio between the observed absorbance and the initial value of absorbance before the commencement of the reaction. Following the Beer-Lambert law and the approximation that only the substrate contributes significantly to the overall absorbance of the solution, the first derivative of \(A_{rel}\) with respect to time is given by the equation,

\[
\frac{\text{d}A_{rel}}{\text{d}t} = \left(\frac{1}{[\text{S}]_0}\right)\frac{\text{d}[\text{S}]}{\text{d}t}
\]

where \([\text{S}]_0\) is the initial substrate concentration. Combining Equations 3 and 2, we obtain the dependence of the enzymatic activity on \(\text{d}A_{rel}/\text{d}t\),

\[
v = -6 \times 10^4 \frac{[\text{S}]_0}{(1/[\text{S}]_0)\text{d}[\text{S}]/\text{d}t}
\]

From every time-resolved trace of \(A_{rel}\), values of \(\text{d}A_{rel}/\text{d}t\) were calculated at time intervals of 0.4 or 4 s. By means of Equation 4, \(\text{d}A_{rel}/\text{d}t\) was converted into \(v\) using a value of 5 mM for \([\text{S}]_0\), the initial concentration used in the experiment. As a result, the time course of the hydrolysis rate \(v\) was determined up to completion of folding. In our experiments, a saturating concentration of substrate was chosen ([S]₀) is more than 10 times higher than the Michaelis constant (Kₘ.). Moreover, a low protein concentration was chosen so that the depletion of substrate was slow enough to maintain such saturating conditions until the completion of folding. In these circumstances, the hydrolysis rate is independent of substrate concentration and can be taken as a direct measure of the concentration of catalytically active enzyme, i.e. enzymatic activity. This procedure allows the time-resolved changes of enzymatic activity to be determined throughout the folding reaction.
Two peaks of Trp38 and Trp64 overlap in the spectrum of the unresolved peak close to 0.22 ppm in the mutant spectrum. The Leu65, and Ala30) and on two resonances in the aromatic region its wild-type value, that of Trp64 undergoes a shift of 0.3 ppm in the P71A mutant, the Leu 65 methyl group resonances (positioned at 0.04 ppm in the wild-type spectrum) move downfield under the chemical shift of the Val20 resonance in the wild-type spectrum. While the chemical shift of Trp 38 maintains its wild-type value, that of Trp64 undergoes a shift of 0.3 ppm in the P71A mutant.

The substitution of alanine for Pro54 is also destabilizing, but to a substantially smaller extent (Table I). The removal of Pro71 also has major consequences for the conformational stability of AcP, as inferred by urea-induced denaturation experiments (Fig. 2). The P71A mutant denatures at urea concentrations substantially lower than wild-type AcP, and the thermodynamic analysis of the urea denaturation curves acquired at equilibrium shows that the free energy of unfolding is reduced by ca. 6 kJ mol\(^{-1}\) (Table I). The substitution of alanine for Pro54 is also destabilizing, but to a substantially smaller extent (Table I).

The one-dimensional \(^1\)H NMR spectra of wild-type AcP and the two mutants were acquired in order to investigate the extent of the structural changes caused by the amino acid substitutions (Fig. 3). The spectra of both mutants exhibit a substantial chemical shift dispersion, indicating that the two mutations do not lead to overall denaturation of AcP. The spectrum of the P71A mutant shows marked differences with respect to the spectrum of wild-type AcP with substantial changes in many of the chemical shifts. As an example, we focus attention on three well resolved and assigned resonances in the aliphatic region (the methyl group resonances of Val\(^{39}\), Leu\(^{65}\), and Ala\(^{30}\)) and on two resonances in the aromatic region (the indole NH proton resonances of Trp\(^{38}\) and Trp\(^{64}\)). In the P71A mutant, the Leu\(^{65}\) methyl group resonances (positioned at 0.04 ppm in the wild-type spectrum) move downfield under the unresolved peak at 0.22 ppm, which contains the methyl group resonances of Ala\(^{30}\) and Val\(^{20}\). At the same time, a peak appears at 0.14 ppm, most probably belonging to Val\(^ {20}\) (the chemical shift of the Val\(^{20}\) resonance in the wild-type spectrum is 0.22 ppm). In the wild-type NMR spectrum there is a peak at 0.27 ppm belonging to Ala\(^{30}\) that is probably shifted under the unresolved peak close to 0.22 ppm in the mutant spectrum. The two peaks of Trp\(^{38}\) and Trp\(^{64}\) overlap in the spectrum of the wild-type protein. While the chemical shift of Trp\(^{38}\) maintains its wild-type value, that of Trp\(^{64}\) undergoes a shift of 0.3 ppm in the P71A mutant.

Val\(^{20}\) is relatively close to Pro\(^{71}\) in the structure (within 7 Å), and more importantly, it is located in the catalytic loop involving residues 15–21. Leu\(^{65}\) and Trp\(^{64}\) do not interact directly with Pro\(^{71}\) (their side chains are more than 8 Å apart), while Ala\(^{30}\) is positioned in the C-terminal region of the first α-helix at the other side of the active site. The fact that the substitution of Pro\(^{71}\) by alanine causes a general change in the NMR spectrum of the protein indicates that this mutation leads to the reorganization of the positions of a number of the protein residues. These structural changes appear extended rather than localized and involve a significant number of residues other than those immediately close to the mutation site. A less dramatic structural change is caused by the mutation of Pro\(^{54}\).

In the spectrum of the P54A mutant, all of the peaks considered in our analysis undergo only minor shifts, and the similarity of the overall spectrum to that of the wild-type protein is far more evident than for the P71A mutant (Fig. 3).

**RESULTS**

**Characterization of the Proline Mutants of AcP**—Two mutants of AcP, each having single amino acid substitutions at the position of the two proline residues (P54A and P71A), were generated in order to investigate the consequences of these substitutions on the structure, stability, and enzymatic activity of AcP. Table I summarizes the steady-state kinetic parameters for the enzymatic activity of wild-type and mutated AcP. The replacement of Pro\(^{54}\) with an alanine residue does not cause significant changes in the substrate binding affinity of the protein and allows nearly 100% of specific activity to persist in the mutated enzyme. This indicates that the amino acid substitution has not caused a significant change in the geometry of the active site. Different arguments apply to the P71A mutant of AcP, since the specific activity is reduced to <1% in this mutant (Table I). The removal of Pro71 also has major consequences for the conformational stability of AcP, as inferred by urea-induced denaturation experiments (Fig. 2). The P71A mutant denatures at urea concentrations substantially lower than wild-type AcP, and the thermodynamic analysis of the urea denaturation curves acquired at equilibrium shows that the free energy of unfolding is reduced by ca. 6 kJ mol\(^{-1}\) (Table I). The substitution of alanine for Pro54 is also destabilizing, but to a substantially smaller extent (Table I).

**TABLE I**

| & | & |
|---|---|---|
| Specific activity\(^a\) | \(K_p\)\(^a\) | \(\Delta G_{H_2O}\)\(^b\) | Urea m value\(^b\) | \(C_m\)\(^b\) (urea) |
| Wild type | 3800 | 0.57 | 21.3 ± 2.0 | 5.3 ± 0.5 | 4.02 ± 0.10 |
| P54A | 3670 | 0.71 | 19.0 ± 2.0 | 5.2 ± 0.5 | 3.66 ± 0.10 |
| P71A | <25 | n.d. | 15.4 ± 2.0 | 5.6 ± 0.6 | 2.76 ± 0.10 |

\(^a\) Data obtained in 0.1 M acetate buffer, pH 5.3, at 25 °C, using benzoylphosphate as a substrate (15).

\(^b\) Data obtained from equilibrium urea denaturation curves acquired in 50 mM acetate buffer, pH 5.5, at 28 °C and analyzed according to Santoro and Bolen (14).

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Fig. 2. Equilibrium urea-induced denaturation of wild-type (filled circles), P54A (open circles), and P71A (filled triangles) AcP. The unfolding experiments were carried out in 50 mM acetate buffer, pH 5.5, at 28 °C. The raw titration curves were converted to plots of the fraction of native protein versus urea concentration using Eq. 1. The thermodynamic parameters of conformational stability obtained from such fitting procedures are summarized in Table I. The thermodynamic parameters of conformational stability obtained from such fitting procedures are summarized in Table I.

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2 van Nuland, N. A. J., unpublished results.
The experiment was repeated under slightly different conditions (in the presence of 3.64% (v/v) hexafluoroisopropyl alcohol) in order to perturb the folding kinetics and therefore investigate the possible existence of any minor phase that could be masked by the presence of the major folding process. It is known that the fast phase of folding of the wild-type protein is substantially accelerated by the addition of hexafluoroisopropyl alcohol, while the slow phase is virtually unaffected (9). The trace reported in Fig. 4 for the P54A mutant is that acquired under these conditions, and the fitting procedure reveals again the presence of a single phase. This allows the conclusion to be drawn that cis-trans isomerization of the Gly53–Pro54 peptide bond is entirely responsible for the slow phase of wild-type AcP folding.

Given that the isomerization of the Gly53–Pro54 peptide bond is the origin of the slow phase of AcP folding, the question naturally arises as to the effect of cis-trans isomerization of the Ser70–Pro71 peptide bond in the folding of this small protein. To investigate this further, the time course of recovery of the enzymatic activity of AcP during folding has been studied. When benzoylphosphate is used as a substrate, the enzymatic activity is proportional to the rate of decay of the substrate-derived absorbance (15). Fig. 5 shows the change of benzoylphosphate absorbance in a series of experiments. Trace 1 derives from a control experiment performed by mixing a 7 M urea solution containing the substrate with a 7 M urea solution containing denatured wild-type AcP. The absorbance does not change significantly with time, indicating that no substrate hydrolysis takes place, a result consistent with the denaturing conditions. Trace 2 also corresponds to a control experiment in which native AcP was mixed with a solution containing the substrate in which the native state of the protein is stable. In this case, a considerable enzymatic activity exists as revealed by the regular decrease of absorbance. In the experiment corresponding to trace 3, AcP denatured in 7 M urea was mixed with a refolding solution containing the substrate. The rate of substrate hydrolysis can be seen to increase as the protein refolds until a maximum and constant level of acylphosphatase activity is reached.

The absorbance traces corresponding to wild-type AcP and the P54A variant were analyzed as described under “Experimental Procedures” to yield plots showing the increase of enzymatic activity upon folding (Fig. 6). These plots can be fitted satisfactorily to a double exponential function, and the kinetic parameters obtained by the fitting procedure are reported in Table II. For the wild-type protein, the rate constant for the fast phase obtained using this procedure is in good agreement with that measured by using fluorescence as a probe to follow the folding reaction. Nevertheless, the rate constant of the slow phase of the development of enzymatic activity is somewhat lower than that measured by fluorescence. Moreover, the relative amplitude of the slow phase appears to be ~48 ± 8% by enzymatic activity measurements, a value considerably different from that of 13 ± 1% calculated by monitoring the folding reaction with intrinsic fluorescence. The enzymatic activity analysis applied to the P54A mutant reveals the presence of a low amplitude slow phase that is not detectable by probing the folding process using fluorescence (compare Figs. 4C and 6B). In addition, this phase is significantly slower than the fluorescence-detected slow phase of the wild-type protein. In the presence of peptidylprolyl isomerase (PPI), an enzyme catalyzing specifically the cis-trans interconversion of peptidylprolyl bonds, this slow phase appears to be dramatically accelerated (PPI, Fig. 6C), identifying Pro^71^, the only proline residue present in the mutant, as the origin of this folding-associated conformational change.

**Fig. 3.** Aliphatic and aromatic regions of the 500-MHz ^1^H NMR spectra of wild-type and mutant AcP molecules. The chemical shift regions between 0.0 and 0.3 and between 9.8 and 10.3 ppm are expanded.
The main kinetic phase in the folding of AcP is associated with the formation of a functional and catalytically active enzyme, confirming previous findings that a large fraction of AcP molecules folds in a cooperative two-state manner (9). The substitution of Pro\(^{54}\) leads to the disappearance of the fluorescence-detected slow phase. This slow phase of folding is accelerated with great efficiency by PPI. It possesses the same relative amplitude (13 \(\pm\) 2%) when the folding process is monitored by fluorescence, far-UV circular dichroism, or time-resolved NMR, and its rate is independent of urea or hexafluoroisopropyl alcohol concentration (9). The acquisition of NMR spectra during folding has shown that the slow phase is characterized by an exponential and cooperative increase in the intensities of the resonances related to the native state and by the concomitant exponential disappearance of the resonances belonging to the unfolded state (9). These data, taken as a whole, indicate that the slow phase detected with optical probes arises from the folding of a small fraction of molecules with the Gly\(^{53}\)–Pro\(^{54}\) peptide bond in a nonnative cis configuration. The cis to trans isomerization appears to have to take place in the unfolded state prior to folding and to be the rate-determining step for the folding of these molecules.

When the folding process is followed by enzymatic activity measurements, the slow phase has a large amplitude in the wild-type protein and does not disappear completely upon substitution of Pro\(^{54}\). However, the residual slow phase in the folding of the P54A mutant revealed by enzymatic activity measurements is still sensitive to PPI, demonstrating that this slow phase results from cis-trans isomerization of the Ser\(^{70}\)–Pro\(^{71}\) peptide bond. The fact that this phase escapes detection with all optical probes and NMR (9) indicates that isomerization of the cis to trans form of this peptide bond takes place in a native-like intermediate and not in the unfolded state as in the case of Pro\(^{54}\). Interestingly, the relative amplitudes of the slow phase detected by enzymatic activity measurements on the P54A mutant (attributable to the cis-trans isomerization of the Ser\(^{70}\)–Pro\(^{71}\) peptide bond) and the slow phase detected using fluorescence measurements for the wild-type protein (attributable to the cis-trans isomerization of the Gly\(^{53}\)–Pro\(^{54}\) peptide bond) are very similar. This indicates that the cis-trans isomerization equilibrium constants in the denatured state of AcP are very similar for the two peptide bonds.

A comprehensive scheme that illustrates the folding behavior of wild-type AcP and that accounts for all the present results is given in Scheme 1. In the unfolded state, we have to include four different conformations, one with both prolines in a trans configuration representing more than 50% of the AcP molecules (U\(_{54t,\_71c}\)), one with both prolines in a cis configuration (U\(_{54c,\_71c}\)), and the other two with both prolines in different configurations (U\(_{54t,\_71c}\) and U\(_{54c,\_71t}\)). Folding can proceed only from the two species possessing the Gly\(^{53}\)–Pro\(^{54}\) peptide bond in a trans configuration, i.e. U\(_{54t,\_71c}\) and U\(_{54c,\_71c}\). In the two conformations of the protein with the Gly\(^{53}\)–Pro\(^{54}\) bond in the cis configuration, i.e. U\(_{54c,\_71c}\) and U\(_{54c,\_71t}\) this peptide bond has to isomerize before the folding process can take place. Only the protein molecules with both prolines in a trans configuration can generate directly the native state of the protein; the U\(_{54c,\_71c}\) species leads to a folded intermediate with the Ser\(^{70}\)–Pro\(^{71}\) bond in the native-like configuration of the protein; the U\(_{54c,\_71t}\) species leads to a folded intermediate with the Ser\(^{70}\)–Pro\(^{71}\) bond in the nonnative configuration, i.e. I\(_{54t,\_71c}\). This can eventually generate the fully native protein by a cis to trans isomerization step. From the kinetic parameters listed in Table II, the rate constants for many of the steps presented in the scheme can be quantified. These are reported in Table III.

It could be argued that the I\(_{54t,\_71c}\) species represents a kinetic trap in the folding process and that completion of folding can occur only if this intermediate unfolds to the U\(_{54t,\_71c}\) species and isomerizes to U\(_{54t,\_71t}\) prior to refolding to the native state. This possibility is, however, very unlikely. In this case, the observed rate constant would be the product of the rate constant \(k_2\) and of the equilibrium constant between U\(_{54t,\_71c}\) and I\(_{54t,\_71c}\). Because this equilibrium constant is extremely small under strong refolding conditions, the observed rate constant would be expected to be many orders of magnitude smaller than that actually observed. Since Pro\(^{71}\) is located in an extended loop with considerable conformational flexibility, there is no reason to suppose that the native-like trans configuration of Pro\(^{71}\) cannot be reached without disrupting the native-like fold of the intermediate. The finding that \(k_3\) is comparable with \(k_4\) represents further evidence that the cis to trans isomerization of Pro\(^{71}\) occurs in a relatively flexible and mobile region of the protein.

Pro\(^{54}\), the sole residue that links the 47–53 \(\beta\)-strand to the 55–66 \(\alpha\)-helix (Fig. 1), appears to be very important in determining the efficiency of AcP folding. The cis configuration of the Gly\(^{53}\)–Pro\(^{54}\) peptide bond appears to be prohibitive for the attainment of a stable native fold. Moreover, the main kinetic phase of folding is considerably decelerated when Pro\(^{54}\) is replaced by alanine, demonstrating that the trans isomer of a proline residue at this position in the amino acid sequence
Protein Folding by Enzymatic Activity Measurements

Table II
Kinetic parameters for folding of wild-type AcP and its proline mutants

Folding of wild type and P54A and P71A mutants of AcP was followed by fluorescence and enzymatic activity measurements. The rate constants ($k_{fast}$, $k_{slow}$) and relative amplitudes ($A_{fast}$, $A_{slow}$) of the fast and slow phases of folding were obtained by fitting the traces of fluorescence and enzymatic activity to double exponential functions. The enzymatic activity analysis could not be performed on the P71A mutant due to the absence of detectable enzymatic activity in this mutant. The reported experimental errors are the S.D. values determined by repeating the experiments several times. The uncertainties of the parameters derived from the enzymatic activity analysis are large, a result of the intrinsically low signal-to-noise ratio of the technique. See “Experimental Procedures” for further details.

|                  | $k_{fast}$ | $A_{fast}$ | $k_{slow}$ | $A_{slow}$ |
|------------------|------------|------------|------------|------------|
|                  | s$^{-1}$   | %          | s$^{-1}$   | %          |
| **Intrinsic fluorescence emission** |            |            |            |            |
| Wild type$^a$    | 0.79 ± 0.10| 86.9 ± 1.0 | 0.076 ± 0.010 | 13.1 ± 1.0 |
| P71A$^a$         | 0.70 ± 0.10| 86.2 ± 1.0 | 0.070 ± 0.010 | 13.8 ± 1.0 |
| P54A$^a$         | 0.17 ± 0.02| 100        |            |            |
| P54A$^a$         | 0.41 ± 0.05| 100        |            |            |
| **Enzymatic activity** |            |            |            |            |
| Wild type$^a$    | 0.75 ± 0.20| 52 ± 10    | 0.033 ± 0.008 | 48 ± 8 |
| P71A$^a$         | ND$^b$     | ND         | ND         | ND         |
| P54A$^b$         | 0.41 ± 0.11| 84 ± 6     | 0.027 ± 0.008 | 16 ± 7 |
| P54A + PPI$^c$   | 0.38 ± 0.10| 100        | 0          | 0          |

$^a$ Conditions: 0.63 M urea, 2.27% HFIP, 50 mM acetate buffer, pH 5.5, 28 °C.
$^b$ Conditions as in Footnote $a$ with the exception of 3.64% HFIP.
$^c$ ND, not determined.
$^d$ Conditions: 2 μM PPI, 0.18 M urea, 3.64% HFIP, 90 mM Tris buffer, pH 7.5, 28 °C.

Fig. 5. Change of the substrate-derived absorbance in the presence of wild-type AcP. The absorbance on the y axis is reported as a fraction of the initial value ($A_{0.1}$). Trace 1, AcP denatured in 7 M urea was diluted into a denaturing and substrate-containing solution. Final conditions were 5 mM benzoylphosphate, 7 M urea in 50 mM acetate buffer, pH 5.5, 28 °C. Trace 2, native AcP was diluted into a refolding and substrate-containing solution. Final conditions as follows: 5 mM benzoylphosphate, 0.63 M urea, 2.27% HFIP in 50 mM acetate buffer, pH 5.5, at 28 °C. Trace 3, AcP denatured in 7 M urea was diluted into a refolding and substrate-containing solution. Final conditions were the same as those of trace 2.

gives rise to efficient folding of AcP. Although the presence of Pro$^{54}$ results in slow folding of a small population of molecules, the overall folding rate, taken as an average, is lower upon substitution of Pro$^{54}$, indicating that proline residues are not necessarily counterproductive with respect to the general efficiency of the folding process. The presence in vivo of enzymes catalyzing specifically the cis-trans interconversion of X-Pro peptide bonds reduces further the problems associated with proline isomerization.

The substitution of Pro$^{71}$ by alanine does not reduce the folding rate of AcP, nor does the cis configuration of this proline appear prohibitive for the attainment of a native-like fold. The substitution does, however, lead to a dramatic reduction of the catalytic efficiency, to significant structural differences, and to a large destabilization of the native state of the protein. Although not located in the active site of the enzyme, Pro$^{71}$ lies in an extended loop that interacts extensively with the 15–21 catalytic loop (Fig. 1). The NMR investigation reveals that the positions of the amino acid residues present in this catalytic loop, as well as in other regions of the molecule, are significantly affected by the substitution of Pro$^{71}$ by alanine. It is probably this structural reorganization upon mutation that gives rise to the destabilization and catalytic inactivation of the enzyme upon mutation. There are considerable analogies between the P71A mutant and the I$^{144 Lys}$–Pro$^{145}$ cis-trans species that forms transiently during folding of AcP. In both cases, the protein has a native-like fold and has structural alterations in the region of the Pro$^{71}$ residue. The rates of folding of both species are similar to those of wild-type AcP, and neither species has acylphosphatase activity. The enzymatic activity of AcP is very sensitive to local structural rearrangements, as demonstrated by the partial loss of catalytic efficiency upon mutation of residues not involved in the active site of the enzyme (11, 16–18). By using steady-state enzymatic activity measurements, a native-like intermediate of ribonuclease T1 with one proline in a nonnative configuration was shown to possess 40% of the enzymatic activity of the fully native protein (19). Hence, monitoring the folding reaction in this manner can constitute a valuable technique for the detection of intermediate conformations that develop late in the folding process.

Folding processes associated with proline isomerization have been studied in detail and are best understood in ribonuclease A and ribonuclease T1. Both proteins contain two cis X-Pro peptide bonds in the native protein. Under strongly refolding conditions, all unfolded molecules of ribonuclease T1 can convert very rapidly into native or native-like intermediates regardless of the configuration of their proline residues (20, 21). Trans to cis isomerization occurs at later stages in the folding of this protein and can be detected and studied because of differences in intrinsic fluorescence emission between these native-like intermediates and the fully native state (19–21). In ribonuclease A, the small fraction of molecules with both prolines in a cis configuration can fold very rapidly to the fully native state. The molecules with either Pro$^{93}$ or Pro$^{144}$, or both, in a nonnative trans configuration can fold to a well structured intermediate, but in this case folding is slower and involves formation of an early, relatively unstructured, intermediate prior to formation of the native-like intermediate (22, 23). Similar results were found in staphylococcal nuclease. In this protein, the folding process can proceed regardless of the cis-trans isomerization state of the Lys$^{16}$–Pro$^{17}$ peptide bond but is faster when this bond is in a cis configuration (24). Interestingly, a cis-trans equilibrium of the Lys$^{16}$–Pro$^{17}$ peptide bond also exists in the native state of staphylococcal nuclease as inferred from magnetization transfer NMR experiments (24).
functions. A of the data points to double (buffer, pH 7.5, 28 °C). The was initiated by diluting AcP denatured in 7 M urea into a refolding and P54A mutant in the presence of PPI. Final conditions were 5 mM • 3-kinase could be attributed to trans–cis proline isomerization steps taking place in a highly unstructured state and limiting the rate of the overall folding process for a small population of molecules (9, 25). In the majority of proteins, the number of slow phases revealed by conventional spectroscopic probes and known to be associated with proline isomerism is smaller than the number of proline residues present in the polypeptide chain (25–29). The ninth fibronectin module of type III, in particular, contains seven proline residues, but only a single slow phase of folding could be attributed to proline isomerization (28). This situation is also seen with AcP, which possesses two proline residues but exhibits only a single slow phase when fluorescence is used to study the folding reaction. The present study reveals, however, that proline isomerization reactions may occur in nearly native conformations and escape detection because of nearly identical spectroscopic properties and rates of formation of native-like intermediates and the fully native protein. “Invisible” reactions of this type can be revealed by double jump experiments specifically designed for this purpose (23). Nevertheless, unfolding of AcP is extremely slow, almost 1 order of magnitude slower than proline isomerization (9). This gives rise to considerable difficulties in studying the proline isomerization steps of AcP folding by double
jump assays. An alternative strategy, involving monitoring the recovery of enzymatic activity during folding, was, however, successful in revealing the proline isomerization steps in AcP folding. Processes of this type are often assumed to be of little significance because they do not interfere with the rate of development of the native fold. This study shows, however, that they can be extremely important in the development of the biologically active conformation of the protein and hence must be included in a complete description of the folding process.

Acknowledgments—We are indebted to Sophie E. Jackson for the generous gift of PPI. We are also very grateful to Paul Webster for technical assistance.

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