Nonsequential Unfolding of the α/β Barrel Protein Indole-3-glycerol-phosphate Synthase†

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Received December 20, 1996; Revised Manuscript Received February 24, 1997⊗

ABSTRACT: The folding of the enzyme indole-3-glycerol-phosphate synthase (IGPS), a member of the $(\alpha/\beta)_8$ fold family, has been studied. At least two folding intermediates have been detected using spectroscopic and activity measurements in combination with gel filtration chromatography. These two intermediates are produced by parallel pathways of a nonsequential unfolding mechanism rather than being consecutive steps in a sequential process. One intermediate can be detected in unfolding experiments because it is kinetically trapped in that conformation, but it is not observed in refolding experiments. It has spectroscopic and hydrodynamic properties very similar to those of the native protein, but it is inactive. The other intermediate could not be characterized because it either aggregates or unfolds under our experimental conditions and could not be isolated chromatographically.

Triosephosphate isomerase (TIM)[1] barrel or $(\alpha/\beta)_8$ proteins comprise about 10% of the enzymes of known structure (Brändén, 1991; Farber & Petsko, 1990). Although some deviations have been described, the $(\alpha/\beta)_8$ fold consists of eight parallel β-strands forming the central cavity of a barrel, surrounded by eight α-helices (Brändén, 1991; Farber, 1993; Farber & Petsko, 1990). The active site is located at the C-terminal end of the β-strands. Proteins sharing this structural motif have a very low sequence identity and catalyze different reactions (Farber & Petsko, 1990; Janecek, 1993). The high flexibility that is required to accommodate substrates with such a variability of structures makes this fold an attractive building block for the design of new enzyme activities (Farber, 1993). Although TIM barrel proteins appear to have a single domain, folding studies revealed that they behave as if there are two folding domains (Eder & Kirschner, 1992; Miles et al., 1982). The N-terminal and C-terminal domains should consist approximately of six and two α/β motifs, respectively. It was proposed that an intermediate is produced on the folding pathway that has a folded N-terminal domain and an unfolded or distorted C-terminal domain (Eder & Kirschner, 1992; Miles et al., 1982). Results from other groups support this folding pathway and suggest that it is shared by the members of the α/β barrel family (Jasanoff et al., 1994; Tsuji et al., 1993).

Indole-3-glycerol-phosphate synthase (IGPS) is a TIM barrel protein that catalyzes the conversion of 1-(2-carboxyphenylamino)-1-deoxyribulose 5′-phosphate (CdRP) to indole-3-glycerol phosphate (IGP). In *Escherichia coli*, IGPS is produced as a bifunctional enzyme with another TIM barrel protein, the *N*-(5′-phosphoribosyl)anthranilate isomerase (PRAI), which catalyzes the previous reaction of the tryptophan biosynthesis. Residues 1−255 and 256−452 of the bienzyme correspond to the IGPS and PRAI domains, respectively. IGPS has an extra helix (α_0) preceding the first strand of the barrel, that is located on top (C-terminus) of the barrel and is part of the active site. There are four residues in the $\beta_8\alpha_8$ loop in a helical conformation (α_8') involved in phosphate binding that are also present, with the same function, in other $(\alpha/\beta)_8$ proteins (Brändén, 1991; Hyde et al., 1988; Wilmanns et al., 1992). The only tryptophan residue of the protein is located in α_0, oriented toward helices 2 and 3, and partially exposed to the solvent (Wilmanns et al., 1992).

MATERIALS AND METHODS

Protein. IGPS was cloned in *E. coli* NM554 without the PRAI domain. The cloned sequence encodes for residues 1−259 of the native bienzyme, plus the tail GSDYKDDDDK at the C-terminal end. This addition had no effect on the enzyme activity. Purification of the enzyme was performed as described by Eberhard et al. (1995) but with an additional final purification step through a Mono Q column. The protein concentration was determined using the Bio-Rad protein assay based on the Bradford method (1976). The final preparation was concentrated, equilibrated in 50 mM potassium phosphate at pH 7.0, and stored at −70 °C. All experiments were performed at 23 °C in 50 mM potassium phosphate, pH 7.0, unless otherwise indicated.

Enzyme Assay. 1-(2-Carboxyphenylamino)-1-deoxyribulose 5′-phosphate (CdRP), the substrate of IGPS, was prepared as described by Kirschner et al. (1987). The assay was initiated by addition of 12 μL of 19 mM CdRP to 800 μL of 0.8 μM IGPS in the indicated buffer. The increase in absorbance at 278 nm was monitored for 30 s, and the activity was expressed in units of μM min^{-1}.

Unfolding and Refolding Experiments. In the experiments referred herein as unfolding experiments, 0.8 μM IGPS (1.2 μM in CD experiments) was incubated for 2 h in the presence of different concentrations of guanidinium chloride (GdmCl) or urea before the fluorescence or CD spectra were recorded.

† M.M.S.d.P was supported by an EC fellowship.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: CdRP, 1-(2-carboxyphenylamino)-1-deoxyribulose 5′- phosphate; GdmCl, guanidinium chloride; IGP, indole-3-glycerol phosphate; IGPS, indole-3-glycerol-phosphate synthase; PRAI, *N*-(5′-phosphoribosyl)anthranilate isomerase; TIM, triosephosphate isomerase.

S0006-2960(96)03133-9 CCC: $14.00

In the experiments referred herein as refolding experiments, IGPS was denatured for 15 h in 6 M GdmCl in 50 mM potassium phosphate at a concentration of 25 μM. Then, the denatured protein was diluted to a concentration of 0.8 μM at the indicated final GdmCl concentrations. The fluorescence spectra were recorded after a 2 h incubation. As will be explained below, these experiments represent equilibrium conditions for all but one of the detected species. Several days of incubation are needed to equilibrate fully this folding intermediate with the other species, which results in protein precipitation.

Spectroscopic Measurements. The change in fluorescence was monitored using an Hitachi F-4500 fluorescence spectrophotometer. Fluorescence emission spectra were recorded at each denaturant concentration using two different excitation wavelengths (λ_{ex}), 280 and 295 nm. The average emission wavelength, $\langle\lambda\rangle$,was calculated using the equation:

$$\langle\lambda\rangle = \frac{\sum_{i=\lambda_1}^{\lambda_N}(F_i\lambda_i)}{\sum_{i=\lambda_1}^{\lambda_N}F_i} \quad (1)$$

where F is the fluorescence intensity and λ the wavelength. This parameter reflects changes in the shape of the spectrum as well as in position. Since it is an integral measurement, it has less error than measurements at a single wavelength (Royer et al., 1993). The $\langle\lambda\rangle$ of tryptophan invariably changes to longer wavelengths upon unfolding.

Unfolding was also monitored by far-UV circular dichroism (CD) using a Jasco 720 spectropolarimeter.

Size-Exclusion Chromatography. IGPS at a final concentration of 9 μM was incubated for 2 h in the presence of the indicated denaturant concentration. Then, 100 μL of sample was loaded onto a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with the same buffer as the sample. The elution was carried out isocratically at a flow rate of 0.67−0.8 mL/min and monitored by the absorbance at 280 nm. The column was connected to a Gilson HPLC system, and the chromatograms were analyzed using the software provided. The peaks were collected, their spectroscopic properties were recorded, and the samples were then rechromatographed under the same conditions. In all cases, the same peak samples were obtained, over 95% pure. The time elapsed between separation and rechromatography of the peaks was longer than 1 h in all cases.

RESULTS

The results in Figure 1 indicate that unfolding of IGPS cannot be described by a two-state mechanism. This is similar to the denaturation of other TIM barrel proteins (Eder & Kirschner, 1992; Jasanoff et al., 1994; Matthews & Crisanti, 1981; Miles et al., 1982). There is a transition at about 1.3 M GdmCl followed by a smooth change of the measured parameters that suggests the presence of other folding species besides the native and denatured forms. The drop in CD signal in the transition is smaller and less cooperative than that observed in fluorescence intensity, indicating that at this GdmCl concentration there must be an intermediate with a larger disruption of tertiary structure

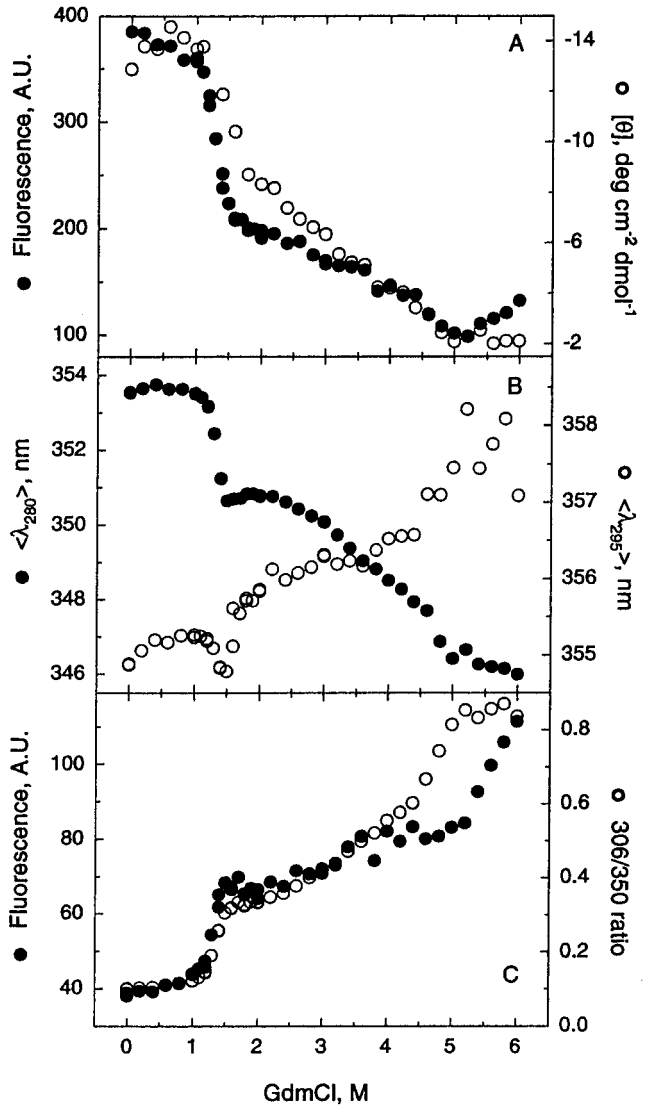

FIGURE 1: IGPS unfolding by GdmCl. Native IGPS was diluted to a final concentration of 0.8 μM protein (1.2 μM in CD experiments) at the indicated GdmCl concentration. (A) Unfolding monitored by fluorescence intensity using λ_{em} 350 nm and λ_{ex} 280 nm (filled symbol), and ellipticity at 222 nm (open symbol). (B) Unfolding monitored by the average emission wavelength, $\langle\lambda\rangle$, using λ_{ex} of 280 nm (filled symbol) and 295 nm (open symbol). (C) Unfolding monitored by fluorescence intensity using λ_{em} 306 nm and λ_{ex} 280 nm (filled symbol), and the 306−350 fluorescence intensity ratio (open symbol).

compared with secondary structure. The presence of this intermediate is also supported by the average emission wavelength ($\langle\lambda\rangle$) data (Figure 1B). The value of the tryptophan $\langle\lambda\rangle$ (λ_{ex} 295 nm) is indicative of its environment, shifting to longer wavelengths upon exposure to the solvent (Royer, 1995). However, the $\langle\lambda\rangle$ value in the transition region is actually decreased, indicating the presence of an intermediate where the tryptophan is more protected from the solvent than it is in the native and denatured conformations. The smooth change in the parameters measured in Figure 1 suggests the presence of a second transition at higher GdmCl concentrations. This is better seen in panels B and C of Figure 1. The $\langle\lambda\rangle$ as well as the 306/350 nm ratio, both using an excitation wavelength of 280 nm, can be regarded as indications of the tyrosine contribution to the emission spectrum. Although the maximum emission wavelength was about 350 nm at all GdmCl concentration used,

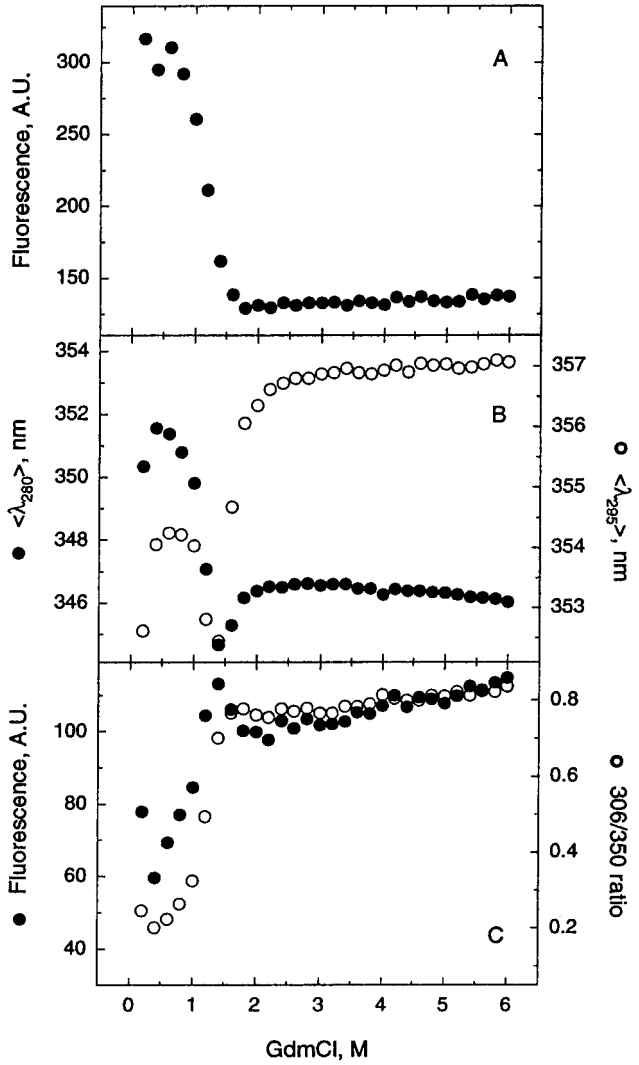

FIGURE 2: IGPS refolding by GdmCl. IGPS denatured in 6 M GdmCl was diluted to a final concentration of 0.8 μM protein at the indicated GdmCl concentration. (A) Refolding monitored by fluorescence intensity using λ_{em} 350 nm and λ_{ex} 280 nm. (B) Refolding monitored by the average emission wavelength, $\langle\lambda\rangle$, using λ_{ex} of 280 nm (filled symbol) and 295 nm (open symbol). (C) Refolding monitored by fluorescence intensity using λ_{em} 306 nm and λ_{ex} 280 nm (filled symbol), and the 306−350 fluorescence intensity ratio (open symbol).

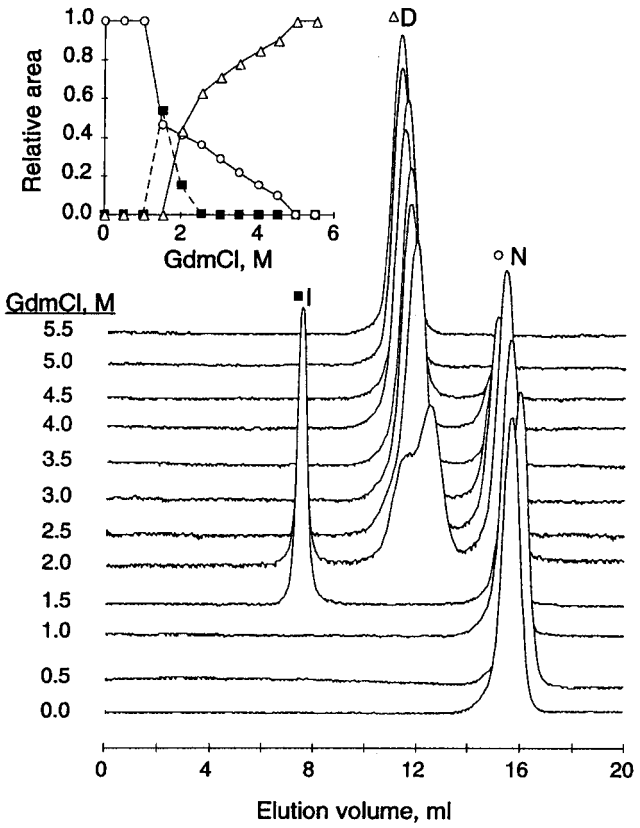

FIGURE 3: IGPS unfolding by GdmCl monitored by gel filtration chromatography. Native IGPS was diluted to a final concentration of 9 μM protein at the indicated GdmCl concentration. The column, Superdex 200 HR 10/30, was equilibrated at the GdmCl concentration shown on the left side. The flow rate was 0.8 or 0.67 (5 and 5.5 M GdmCl) mL/min and was monitored by the absorbance at 280 nm. Inset: relative area of peaks for N, I, and D forms shown in the chromatograms.

an increase in fluorescence intensity in the tyrosine emission region (300−320 nm) upon denaturation is observed (Figure 1C). These results suggest the presence of a different folding intermediate between 1.8 and 5 M GdmCl, approximately, which disappears in a noncooperative way. One explanation for the absence of tyrosine emission in proteins is energy transfer from tyrosine to tryptophan residues (Lakowicz, 1983). If this is a significant factor for IGPS, the increase in tyrosine emission would indicate that the single tryptophan residue, located in helix α_0, has moved away from the barrel where the closest tyrosines are located.

Only about 80% of the protein regained native conformation following renaturation after complete denaturation in 6 M GdmCl. Despite the apparent two-state behavior suggested by the fluorescence intensity, the $\langle\lambda\rangle$ data reveal that this is not the case (Figure 2). Additional species are present in the transition region, as indicated by the decrease in the value of this parameter. Moreover, the observed decrease is larger than in unfolding experiments, indicating that at least some of the species present are more populated in refolding experiments. The second transition is not present, indicating that unfolding and refolding do not go through the same intermediate species.

Further insight in the unfolding process can be obtained by gel filtration chromatography (Uversky, 1993). The elution profiles shown in Figure 3 correlate very well with the CD and fluorescence data (Figure 1). The first transition observed above corresponds to the appearance of a peak that is present only at the transition region and elutes in the void volume. Thus, it probably reflects aggregation of a folding intermediate, referred herein as I. Even though it represents about 50% of the total protein of the chromatogram, aggregation represents a much lower percentage at the protein concentration used in the experiments shown in Figure 1 (see below and Figure 7). At higher GdmCl concentrations, this peak is replaced by another that has an intermediate elution volume, between the native and the void volume. This intermediate peak corresponds to the denatured protein because it is the only one present in 6 M GdmCl. However, at 2 M GdmCl it appears as a double peak, which could result from the presence of lower order association forms of I as the stability of the aggregate decreases. The smooth changes observed in CD and fluorescence signals between 1.8 and 5 M GdmCl are explained by the continuous replacement of the native protein peak by the one for the denatured protein. The apparent second transition observed at about 4.5 M GdmCl coincides with the disappearance of

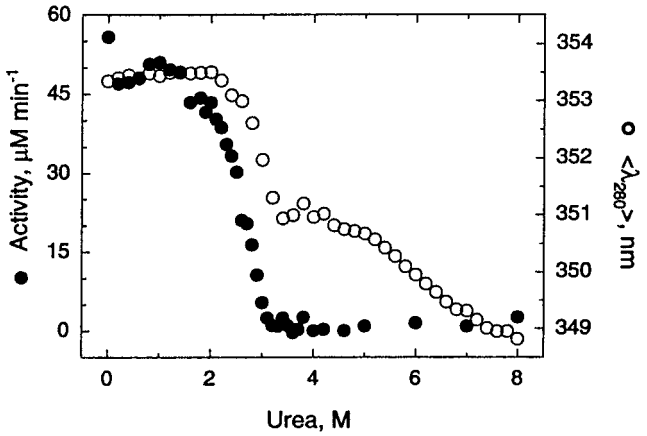

FIGURE 4: IGPS unfolding by urea. Native IGPS was diluted to a final concentration of 0.8 μM protein at the indicated urea concentration. Unfolding was monitored by enzyme activity measurements (filled symbol), and by the average emission wavelength, $\langle\lambda\rangle$, using a λ_{ex} of 280 nm (open symbol).

the native peak. It seems unlikely that approximately half of the native protein would be involved in a very cooperative transition, at about 1.3 M GdmCl, whereas the remaining would unfold in a noncooperative way. It is more probable that a second intermediate with the same hydrodynamic properties as the native protein would be produced after the first transition. This intermediate would then unfold in a noncooperative way. The peak corresponding to native and, presumably, the second intermediate forms is not present at 3 M GdmCl when the protein was previously denatured in 6 M GdmCl (not shown), which explains the differences between unfolding and refolding shown in Figures 1 and 2.

To determine unambiguously if there is a folding intermediate with the same hydrodynamic volume as the native protein, we performed activity measurements in the presence of urea. GdmCl could not be used because IGPS loses its activity when the ionic strength is increased (not shown). Figure 4 shows the unfolding of IGPS by urea monitored by activity and fluorescence. The urea-unfolding profiles obtained with fluorescence measurements are like those obtained in the presence of GdmCl but shifted to higher denaturant concentration. Thus, unfolding in the presence of urea or GdmCl can be considered as equivalent with regard to the folding intermediate produced. The activity is lost in a cooperative way with a two-state behavior, which takes place at a lower urea concentration than for the drop in fluorescence. The species present at different urea concentrations were separated by gel filtration chromatography, and their activity was measured (Figure 5). Only the peak corresponding to the native protein is active. Even though this peak is present up to 6 M urea, no activity was detected at 3 M. These results establish the presence of an inactive folding intermediate that has the same hydrodynamic volume as the native protein, referred herein as NL. In Figure 5B are shown the species detected by gel filtration chromatography in combination with activity measurements.

The different species present during the unfolding of IGPS were further characterized. The peaks that were separated by gel filtration chromatography at different GdmCl concentrations were collected and their CD and fluorescence spectra recorded (Figure 6). The three main peaks show distinctive spectroscopic properties. The contribution of tyrosine emission in the denatured form is clear when spectra

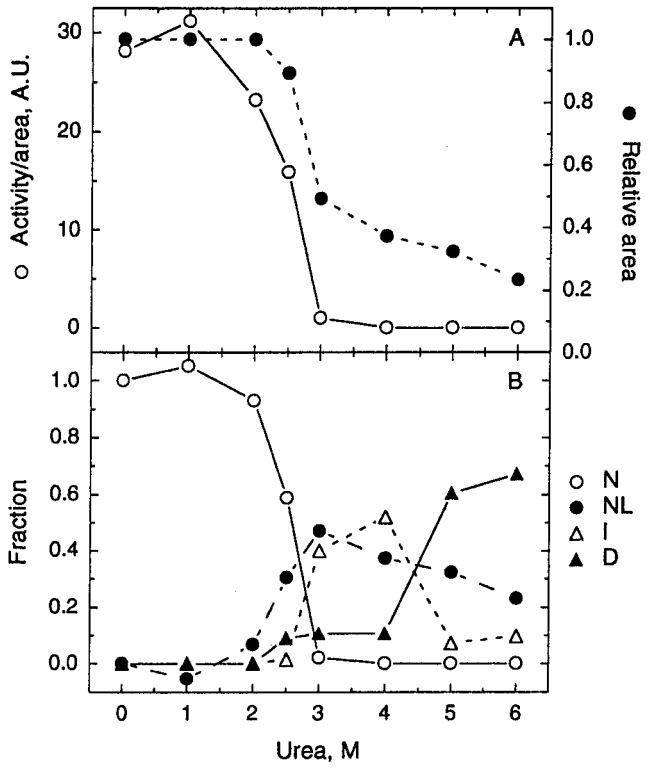

FIGURE 5: IGPS unfolding by urea monitored by gel filtration chromatography. Native IGPS was diluted to a final concentration of 9 μM protein at the indicated urea concentration. The eluted peaks were collected, and their enzyme activity was measured. (A) Enzyme activity of the peak with the same elution volume as the native protein, normalized by its area (open symbol), and relative area of the peak (filled symbol). (B) Fraction of the different species present at the indicated urea concentration. The fractions of I and D correspond to the values of their relative areas, whereas the fractions of N and NL were calculated as the active and inactive fraction, respectively, of the peak area shown in panel A.

obtained with λ_{ex} at 280 and 295 nm are compared. The blue shift of the aggregated form of I indicates a shielding of the tryptophan from the solvent and explains the drop in $\langle\lambda\rangle$. The NL conformation (above 2 M GdmCl) and the native protein both have almost identical CD and fluorescence spectra. There is only a slight increase in emission at 300−320 nm (λ_{ex} 280 nm) in NL compare with the native protein. The fraction of each form (peak) present in unfolding and refolding experiments can be calculated from the $\langle\lambda\rangle$ obtained for each peak according to eq 2:

$$\langle\lambda_{280}\rangle = f_N\langle\lambda^N_{280}\rangle + f_I\langle\lambda^I_{280}\rangle + (1 - f_N - f_I)\langle\lambda^D_{280}\rangle$$
$$\langle\lambda_{295}\rangle = f_N\langle\lambda^N_{295}\rangle + f_I\langle\lambda^I_{295}\rangle + (1 - f_N - f_I)\langle\lambda^D_{295}\rangle \quad (2)$$

where f is the fraction of native (N, it also includes NL), intermediate (I), and denatured (D) forms, and the subscripts 280 and 295 refer to the excitation wavelength used. It is assumed that $f_N + f_I + f_D = 1$. The parameters $\langle\lambda\rangle$ were used because they are integral values and independent of protein concentration, and so contain less experimental error. The results of these calculations (Figure 7) correlate well with those observed by gel filtration. As noted above, aggregation of I is not an important factor in unfolding experiments, which is expected because of the lower protein concentration used in these experiments. Moreover, since I is partitioned between aggregation and unfolding (see below),

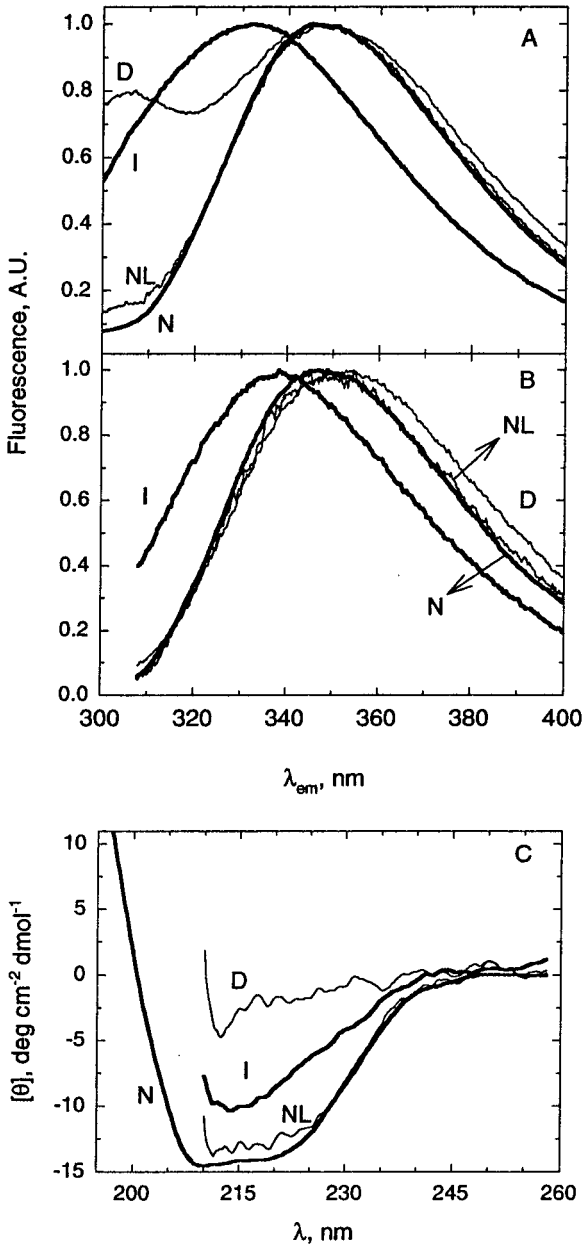

FIGURE 6: Spectroscopic characterization of the different folding species. Average fluorescence (A and B) and CD (C) spectra of folding species isolated by gel filtration chromatography in the presence of GdmCl. The spectra corresponding to N were recorded in the absence of GdmCl whereas those corresponding to NL were recorded at GdmCl concentrations higher than 2 M. (A) Fluorescence spectra using a λ_{ex} of 280 nm. (B) Fluorescence spectra using a λ_{ex} of 295 nm. (C) CD spectra.

the decrease in the fraction of aggregated I should be accompanied by an increase in the fraction of D, as can be observed by comparing Figures 3 and 7. In refolding experiments, however, the fraction of aggregated I is more prominent. It should be mentioned that we have characterized the aggregated form of I because it is the form that can be isolated chromatographically. Since the soluble form of I was not characterized, it is possible that some I is included with some of the other forms in Figure 7.

DISCUSSION

At least four different species can be identified in the unfolding pathway of IGPS. The results have been interpreted according to the scheme shown in Figure 8,

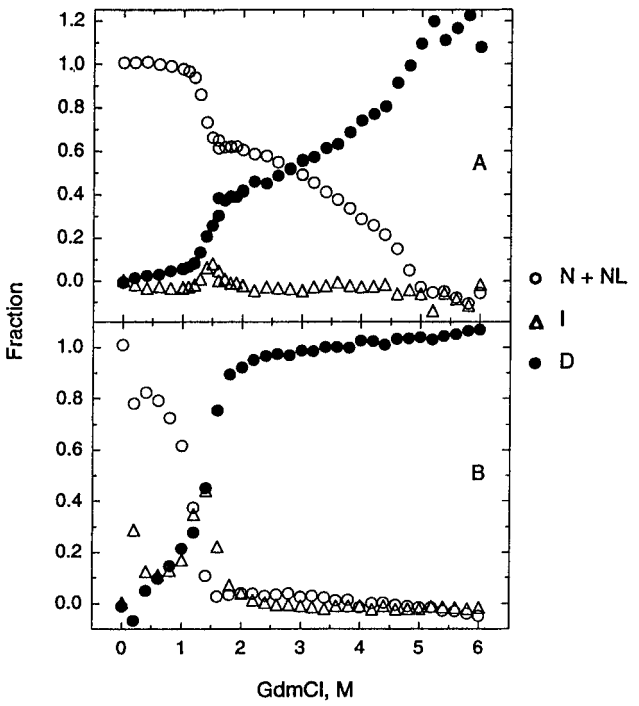

FIGURE 7: Fraction of folding species present in unfolding and refolding experiments. The fraction of N + NL, I, and D species under the experimental conditions shown in Figures 1 (A) and 2 (B) were calculated according to eq 2 under Results. The $\langle\lambda_{280}\rangle$ and $\langle\lambda_{295}\rangle$ values were obtained from the spectra shown in Figure 6.

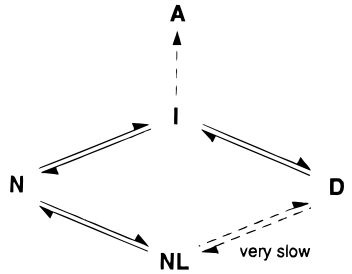

FIGURE 8: Proposed folding pathway.

which we believe is the simplest mechanism that can explain our results.

Under mild denaturation conditions, IGPS loses its activity to produce an intermediate species (NL) with very similar spectroscopic and hydrodynamic properties to those of the native form (N). Simultaneously or at slightly higher denaturant concentrations, a different intermediate (I) is produced. The existence of I is indicated mainly from the presence of a form characterized by a large hydrodynamic volume and a less solvent-accessible tryptophan compared with the native protein. This form probably corresponds to an aggregate of I, which produces a drop in fluorescence and CD signals. I is unstable, and it either aggregates or unfolds completely under our experimental conditions. Thus, its characterization was not possible. As discussed below, NL must be a different intermediate species, and not just the soluble form of I. At higher denaturant concentrations, the only forms present are NL and denatured (D) protein, and they coexist for a wide range of denaturant concentrations. Interconversion between these species is slow because they could be separated by gel filtration chromatography.

Formation of intermediates is nonsequential rather than sequential. The nonsequential mechanism is indicated by

several results. Accumulation of I, as detected by the presence of its aggregated form, takes place only in a narrow range of denaturant concentration. However, NL is present at lower and higher denaturant concentrations, which is difficult to explain assuming consecutive steps. Rechromatography of isolated peaks produced single peaks rather than a mixture of species (not shown) as would be expected from a sequential mechanism. In a sequential model, it is expected that the results in unfolding and refolding experiments would be the same, rather than those presented here. These findings support the nonsequential model and the intermediates NL and I being different rather than being the soluble and aggregated states of the same form.

A native-like conformation is kinetically trapped in unfolding conditions. The fluorescence spectra observed above 2 M GdmCl in refolding experiments are like that of the denatured state in unfolding experiments, indicating that NL is not present over that range of concentrations. This was confirmed by gel filtration chromatography (not shown). Accordingly, a greater population of I was observed at the transition in refolding experiments. The apparently different pathways for unfolding and refolding can be explained if direct unfolding of NL is very slow or nonexistent. When native protein is placed in unfolding conditions, there is a partition between I and NL species where the velocity of formation of each one depends on the denaturant concentration. Under such unfolding conditions, formation of folded protein can be considered negligible so that the fraction of NL is trapped in that conformation. Formation of I is faster at increasing denaturant concentrations. Again, the I form produced is partitioned between aggregation and unfolding. Aggregation takes place only over a narrow range of denaturant concentrations, where it is faster than unfolding. On the contrary, when unfolded protein is placed in refolding conditions, the only folding pathway available is via I. Thus, NL can be formed only after I and N are produced, which occurs at low denaturant concentrations. A nonsequential mechanism was also proposed for PRAI, another TIM barrel protein, by Jasanoff et al. (1994). The model they proposed was a simplification of the one presented here. The main difference between our results and those from other $(\alpha/\beta)_8$ proteins is that, in the scheme shown in Figure 8, the values of the rate constants involved in the NL $\leftrightarrow$ U equilibrium are assumed to be much smaller than those of the other equilibria. Otherwise, the same unfolding and refolding profiles would be expected. It should be pointed out that under our experimental conditions, NL is trapped after the partition of N between I and NL rather than being fully equilibrated with all the other species present.

The native-like (NL) conformation described here could be the folding intermediate detected in other TIM barrel proteins. It is thought that in TIM barrel proteins there is a folding intermediate in which the C-terminal portion of the protein, comprising helices 6−8 and strands 7−8, is disorganized (Eder & Kirschner, 1992; Miles et al., 1982). Part of the IGPS active site resides in this area (Wilmanns et al., 1992), including the binding site of the phosphate moiety of the substrate, located at the N terminus of helix α_8'. Thus, the activity could be lost by a minor structural distortion of this region without affecting the hydrodynamic and CD properties in a significant way. IGPS fluorescence cannot be considered as a good probe for structural changes in this region because the tryptophan residue is located on top of helices 2 and 3, on the opposite side of the barrel. Nevertheless, the slight increase in the tyrosine contribution to the fluorescence spectrum indicates a less compact tertiary structure. The most significant difference detected between NL and native protein is that NL is inactive, which suggests that NL may correspond to the folding intermediate observed in other TIM barrels (Eder & Kirschner, 1992; Jasanoff et al., 1994; Miles et al., 1982; Tsuji et al., 1993).

ACKNOWLEDGMENT

We thank Dr. J. Blackburn for providing us with the clone and expression system for IGPS.

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BI963133Z