Detection of an Intermediate in the Folding of the $(\beta\alpha)_8$-Barrel *N*-(5′-Phosphoribosyl)anthranilate Isomerase from *Escherichia coli*†

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ABSTRACT: We have used thermodynamic and kinetic techniques to monitor the guanidinium chloride induced (GdmCl-induced) denaturation of *N*-(5′-phosphoribosyl)anthranilate isomerase from *Escherichia coli* (ePRAI). Although CD-monitored equilibrium denaturation curves are consistent with cooperative unfolding of the protein centered at 1.45 M GdmCl, fluorescence readings drop by over 25% in the region preceding the CD-monitored transition, suggesting non-two-state behavior. Kinetics experiments measure a slow relaxation rate with negative fluorescence amplitude when protein is diluted from 0 to 0.5 M GdmCl, corroborating results from equilibrium conditions. Detection of several unfolding and refolding rates in final GdmCl concentrations from 0 to 5.0 M indicates the presence of at least one intermediate along unfolding and refolding pathways. GdmCl dependence of the relaxation rates can be explained most easily by a nonsequential mechanism for ePRAI unfolding, though a sequential mechanism cannot be ruled out. The data corroborate the fragment complementation studies of Eder and Kirschner [Eder, J., & Kischner, K. (1992) *Biochemistry 31*, 3617–3625], which are consistent with unfolding of the C-terminal portion of a yeast-derived PRAI in its folding intermediate. In ePRAI, such partial unfolding would expose W391 to quenching by solvent molecules; W356, ePRAI's other tryptophan, is buried in the hydrophobic core and is unlikely to be affected by local changes in structure. A C-terminally unfolded folding intermediate has been demonstrated in the folding of tryptophan synthase (α-subunit), a related $\beta\alpha$-barrel enzyme. In the context of previous studies, our results suggest but do not prove that the PRAI folding intermediate has similar structure to the tryptophan synthase intermediate; the data are discussed with speculation as to a possibly general model for $\beta\alpha$-barrel folding.

Over 10% of enzymes with known structures belong to the class of protein folds known as $\beta\alpha$-barrels, or TIM-barrels,[1] first noticed in the crystal structure of triose phosphate isomerase (Banner et al., 1975). *N*-(5′-Phosphoribosyl)-anthranilate isomerase (PRAI) is a small $(\beta\alpha)_8$-barrel protein which catalyzes the third step in the biosynthesis of tryptophan from chorismate in some plants and microorganisms. The *Saccharomyces cerevisiae* enzyme (yPRAI) has been the subject of several studies focusing on the association of individual $\beta\alpha$-units in the wild-type enzyme and engineered variants (Luger et al., 1989; Eder, 1991; Eder & Kirschner, 1992; Eder & Wilmanns, 1992). Equilibrium denaturation of yPRAI shows that the protein follows a biphasic folding transition, as has been observed for other proteins in the $\beta\alpha$-barrel superfamily (Matthews & Crisanti, 1981; Eder, 1991). The three states most probably correspond to the fully folded and fully denatured forms of the protein and to an intermediate in which the N-terminal six β-strands and five α-helices are assembled into a native-like conformation while the C-terminal portion is partially unfolded (Eder & Kirschner, 1992). Similar results have been reported for the α-subunit of tryptophan synthase (Miles et al., 1982; Beasty & Matthews, 1985; Tsuji et al., 1993), which exhibits close structural homology and sequence similarity with PRAI in regions of catalytic importance (Farber & Petsko, 1990; Wilmanns et al., 1991).

Escherichia coli PRAI (ePRAI) shares 28% sequence identity and 46% sequence homology with yPRAI (Eder & Kirschner, 1992). It is produced as a covalently linked bienzyme with indole-3-glycerol-phosphate synthase, another $\beta\alpha$-barrel enzyme, which catalyzes the subsequent step in tryptophan synthesis. The crystal structure of ePRAI·IGPS has been solved using multiple isomorphous replacement and later refined to 2.0-Å resolution (Priestle et al., 1987; Wilmanns et al., 1992). Both exhibit modified $(\beta\alpha)_8$-folds with an additional helical turn (helix 8′) in the loop connecting strand 8 to helix 8. ePRAI differs further from $(\beta\alpha)_8$ ideality in the degeneracy of its helix 5, which is disrupted by P366 (residue numbers for ePRAI correspond to positions in the bienzyme) and does not adopt helical geometry. The ePRAI domain of the PRAI·IGPS bienzyme has been cloned, expressed, and purified as a monoenzyme for this study. All of ePRAI's five peptide–proline bonds are in the *trans* conformation in the crystal structure. Two tryptophans, W356, partially exposed on the catalytic face of the barrel, and W391, mostly buried between helices 6 and 7 on the perimeter of the barrel, account for a 50% drop in fluorescence upon unfolding of the protein. ePRAI contains no disulfide bonds and can be reversibly unfolded using guanidinium chloride (GdmCl) and urea, making it a suitable subject for analysis of protein folding.

In this investigation, ePRAI has been studied by thermally and chemically induced equilibrium denaturation and by stopped-flow kinetic methods. Though they do not prove a specific folding mechanism, equilibrium unfolding data are consistent with denaturation of the C-terminal portion of the protein at low [GdmCl], followed by concerted unfolding of the partially folded intermediate. Several unfolding and refolding phases complicate analysis of the kinetics data, but are also consistent with the N- and C-terminal folding domains

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[1] TIM = triose phosphate isomerase.

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model. The data corroborate the results of previous experiments on $\beta\alpha$-barrels and lend support to the suggestion that members of this important class of proteins share a common folding pathway.

METHODS AND MATERIALS

Preparation of Protein Samples and Buffers. ePRAI has been cloned and overexpressed in *E. coli* SG200-50 strain without its IGPS domain (which forms a covalently linked bienzyme with ePRAI in naturally occurring bacteria) and was purified from inclusion bodies as described previously for yPRAI (Luger et al., 1989). All experiments were performed in 50 mM potassium phosphate, pH 7.4 ($[KH_2PO_4]/[K_2HPO_4]$ = 0.511), unless otherwise noted. Experimental concentrations were determined using absorbance spectroscopy with a molar extinction coefficient of 24 100 M^{-1} cm^{-1} for ePRAI at 280 nm. Denaturant solutions (urea and guanidinium chloride) were prepared gravimetrically using volumetric flasks or by serial dilution of a gravimetrically prepared stock. Cyclophilin concentration (for refolding experiments) was determined using a molar extinction coefficient of 14 000 M^{-1} cm^{-1}.

Equilibrium Measurements. Equilibrium denaturation of ePRAI was performed using GdmCl and urea as denaturants, and the change in fluorescence was monitored using a Perkin-Elmer LS5B luminescence spectrometer. Excitation and emission wavelengths were set to 278 and 325 nm, respectively, at which there is maximum change in the fluorescence of ePRAI upon unfolding. Unfolding was also monitored by near- and far-UV circular dichroism (CD), using a Jasco 720 spectropolarimeter equipped with a Neslab temperature controller. In addition, ePRAI was subjected to temperatures ranging from 5 to 95 °C and unfolding followed by CD and differential scanning microcalorimetry (DSM), using a Microcal MC-2 differential scanning microcalorimeter. All experiments except those involving thermal denaturation were theromostated to 25 °C.

Kinetic Measurements. Stopped-flow fluorescence experiments were performed on a Perkin-Elmer MPF-44B fluorescence spectrometer equipped with a rapid-mixing head. The apparatus has a built-in dead time of 30 ms. Denaturation of native protein and refolding of unfolded protein were initiated by rapid 1:10 dilution of protein samples into denaturing and renaturing concentrations of GdmCl, respectively. For renaturation experiments, protein was denatured in 2.75 M GdmCl for at least 1 h before refolding. The protein is completely denatured at 2.75 M GdmCl as judged by fluorescence and CD. The susceptibility of slow-refolding phases to catalysis by cyclophilin was studied by 1:10 dilution of denatured ePRAI (4 μM) into refolding buffer containing 2 μM cyclophilin. Owing to the sensitivity of cyclophilin to GdmCl, urea was used in this experiment at a final concentration of 0.4 M.

Data Analysis. Spectroscopic data processing and unweighted least-squares curve fitting were performed using the Kaleidagraph software package (Abelbeck). Sigmoidal equilibrium denaturation curves were fit to an equation for thermodynamic transition in a two-state system:

$$O = \{(a_N + b_N[D]) + [(a_U + b_U[D])\exp[(m_{U\text{-}F}[D] - \Delta G_{U\text{-}F}^{H_2O})/RT]]\}/\{1 + \exp[(m_{U\text{-}F}[D] - \Delta G_{U\text{-}F}^{H_2O})/RT]\}$$

where O is the measured value of the observable; [D] is the denaturant concentration; a_N, b_N, a_U, and b_U are the intercepts and slopes of the baselines at low and high [D], respectively; $\Delta G_{U\text{-}F}^{H_2O}$ is the free energy change upon unfolding of ePRAI in water; and $m_{U\text{-}F}$ is the empirical m-value (Tanford, 1968; Pace, 1986), which reflects the sensitivity of the protein's stability to denaturant. Simulated relaxation rates at different GdmCl concentrations were solved as the eigenvalues of a matrix of individual rate constants between three interconverting states. Eigenvalues of a microscopic transition rate matrix correspond to macroscopically observed rates in an equilibrating system. The program Mathematica (Wolfram Research) was used for the linear algebra calculations, and exponential dependencies of individual rate constants on [GdmCl] (kinetic m-values) were assumed. Fluorescence traces from kinetic experiments were fit to multiple-exponential decays.

Calorimetry data was displayed using the Microcal Origin software. Analysis of the ePRAI and other $\beta\alpha$-barrel crystal structures used the molecular graphics application InsightII, and ribbon diagrams were generated using the program Molscript. Solvent-accessible surface areas were calculated according to the algorithm of Lee and Richards (1971) with a probe radius of 1.4 Å. Errors for reported quantities are either those generated by Kaleidagraph for curve fits or calculated standard errors of the mean for multiple measurements.

RESULTS

Equilibrium Denaturation. Normalized fluorescence and CD-monitored GdmCl-induced denaturation curves for ePRAI are shown as Figure 1A. The near- and far-UV CD denaturation curves fit well to an equation modeling a two-state thermodynamic equilibrium, if negatively sloping baselines are allowed. The midpoint of GdmCl denaturation for these curves at 1.45 ± 0.02 M. Fluorescence readings drop smoothly from an initial value at 0 M GdmCl until 2.0 M GdmCl, which is also the upper [GdmCl] limit for the CD-monitored transitions. At 2.0 M GdmCl, the residual fluorescence is approximately 50% of the initial amplitude. Approximately half of the fluorescence change on unfolding (~25% overall fluorescence) is lost by 1.0 M GdmCl, which precedes the CD-monitored transitions. Similar results are obtained when urea is used as the denaturant (data not shown).

If the fluorescence and CD decreases before 1.0 M GdmCl are interpreted structurally, they are consistent with (but do not unambiguously imply) unfolding in the intermediate of the C-terminal portion of ePRAI, which contains one of the two tryptophan residues. In particular, dissociation or partial unfolding of the C-terminal two $\beta\alpha$-units could expose W391, normally buried between helices 6 and 7, to quenching by solvent, as well as account for the observed ~20% drop in the far-UV CD signal (which may reflect loss of secondary structure) in the 0–1.0 M GdmCl range (Figure 2). This interpretation would also account for the m-value of 3.7 ± 0.3 kcal mol^{-2} observed for the cooperative CD-monitored transitions, which is significantly lower than a theoretical m-value (6.0) calculated on the basis of free energies of transfer of side chains from water to GdmCl solutions [Pace, 1986; values from Nozaki and Tanford (1970)]. The rationale for speculation as to the structure of the unfolding intermediate is discussed in more detail below.

The thermal denaturation of ePRAI in 50 mM potassium phosphate (pH 7.4) has been monitored by CD (Figure 1B) and differential scanning microcalorimetry (data not shown). Although both methods detect a fairly sharp, symmetric unfolding transition with a T_m of 51 °C, the analysis is complicated by a tendency of the protein to aggregate at

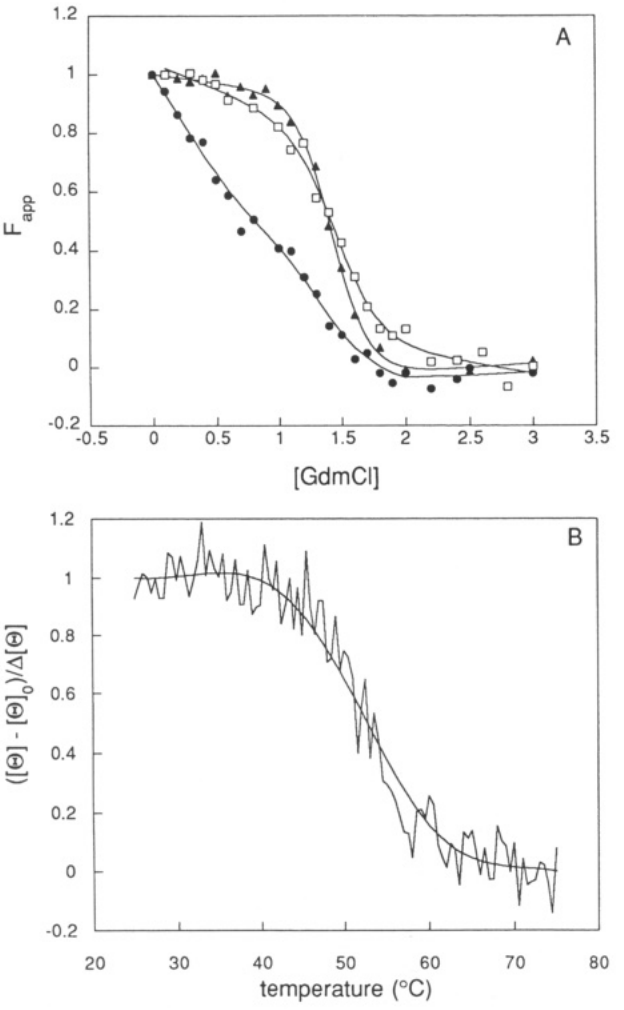

FIGURE 1: (A) Comparison of F_{app}, the apparent fraction of folded ePRAI, normalized to the amplitude of the fluorescence change in 0–3 M GdmCl, as monitored by (□) CD at 278 nm, (▲) CD at 222 nm, and (■) fluorescence with excitation and emission wavelengths set at 278 and 325 nm, respectively. CD data have been fitted to a two-state transition curve, and fluorescence data have been fitted to a smooth function. (B) Thermal denaturation of ePRAI as monitored by CD at 202 nm and normalized to the amplitude of the transition. Raw and smoothed data are shown.

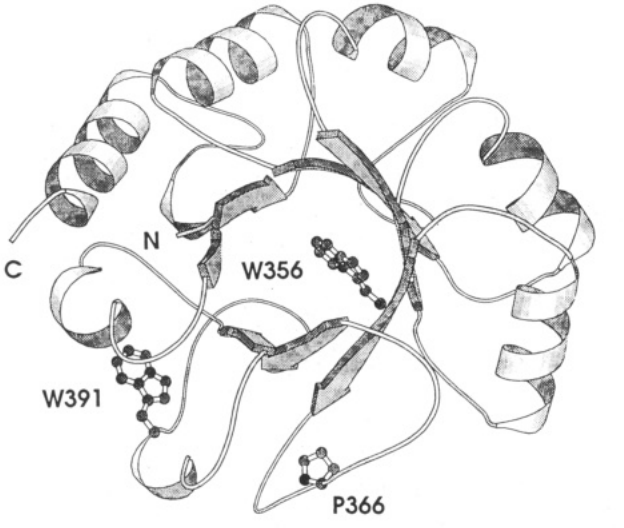

FIGURE 2: Ribbon diagram of the structure of ePRAI (Wilmanns et al., 1992) showing W356, P366, and W391. N- and C-termini are labeled.

temperatures above the T_m. Aggregation may be reversed by addition of 6 M GdmCl. The far-UV spectrum of ePRAI at 75 °C indicates that the thermally denatured form of the protein may have considerable residual secondary structure, possibly stabilized by the aggregation, though no significant optical rotation is detectable at high temperature in the near-UV range. Reliable values for thermodynamic parameters of unfolding cannot be obtained under these conditions, but the thermal denaturation profiles indicate that any local unfolding transition that occurs prior to the global transition at 51 °C is likely to be uncooperative, since there is no significant perturbation of C_p before the global transition.

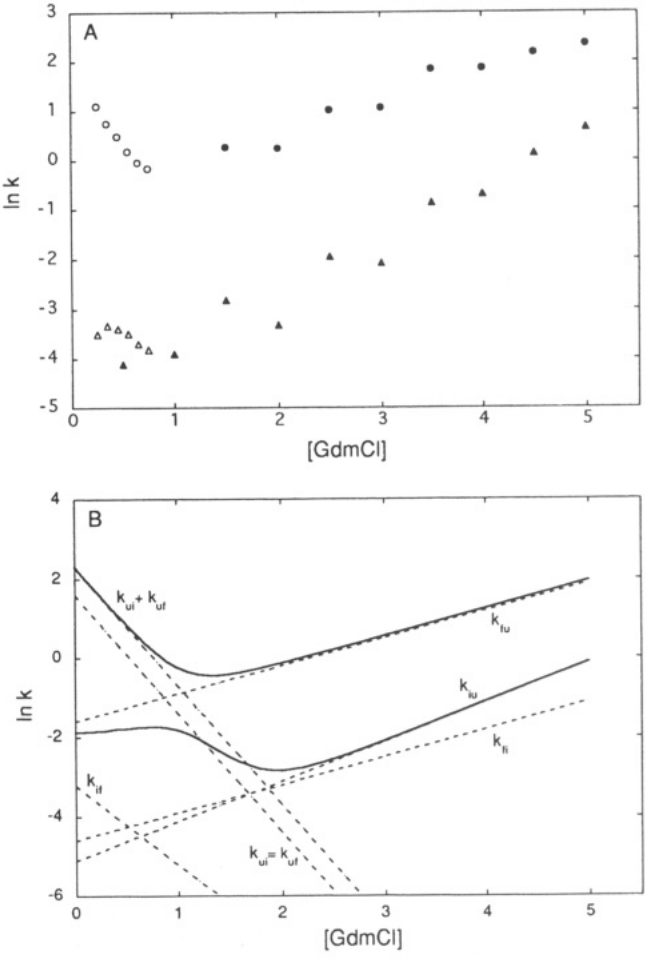

FIGURE 3: (A) Rate constants obtained from unfolding (filled symbols) and refolding (open symbols) kinetics experiments. Circles denote the faster observed relaxation rate, and triangles denote the slower rate. (B) Simulated data based on a nonsequential three-state model for ePRAI folding. Solid lines are simulated relaxation rates. Dashed lines are individual rate constants (x = [GdmCl]); $k_{ui} = k_{uf} = 5e^{-3x}$; $k_{fi} = 0.01e^{0.7x}$; $k_{fu} = 0.2e^{0.7x}$; $k_{if} = 0.04e^{-2x}$; $k_{iu} = 0.006e^{x}$.

Unfolding Kinetics. The kinetics of the unfolding of ePRAI has been studied in the [GdmCl] range 1.5–5.0 M (Figure 3A). At these concentrations the fluorescence decay follows a double-exponential decay as a function of time. Both decay amplitudes correspond to a drop in fluorescence, with a fast phase (10 s^{-1} in 5.0 M GdmCl) accounting for 2–5 times the amplitude (increasing with [GdmCl]) of a slower phase (1.9 s^{-1}). Unfolding kinetics has also been monitored at 0.5 and 1.0 M GdmCl, which lie below the CD-monitored equilibrium unfolding transition region. At these concentrations, there is significant fluorescence decay with a single observable rate constant (0.02 s^{-1} at 1.0 M GdmCl). This is consistent with equilibrium denaturation studies in supporting three-state unfolding of ePRAI, assuming that the unfolding relaxation rate observed in 0.5–1.0 M GdmCl is due predominantly to unfolding of the native protein to the partially folded

intermediate. The existence of further unfolding intermediates or parallel pathways unresolvable by fluorescence or dissipated within the 30-ms dead time, however, cannot be ruled out.

Unfolding of ePRAI in 4.0 M GdmCl and 40 mM potassium phosphate (pH 7.4) has been conducted in the presence of 1% (vol/vol) trifluoroethanol (TFE) and 8% glycerol. TFE has been shown to stabilize helical structure in polypeptides (Doty et al., 1954; Liebes et al., 1975), though addition of 1% TFE has a negligible effect on the far-UV CD spectrum of ePRAI. TFE is found to enhance both unfolding rates, suggesting that neither step involves concerted loss of specifically helical secondary structure. Glycerol (8%) slows both rates roughly equivalently, suggesting that both unfolding phases may involve diffusive processes, as observed for the rate-determining relaxation phase of the α-subunit of tryptophan synthase (Chrunyk & Matthews, 1990). Rigorous analysis of the effects of TFE and glycerol on ePRAI folding depends on quantitation of the native and intermediate-state free energy perturbations introduced by the presence of the solvents; our solvent studies are merely incidental.

Refolding Kinetics. Denatured ePRAI has been refolded in 0.25–0.75 M final [GdmCl] (Figure 3A). Two decay phases are observed with rate constants of 0.030 and 3.0 s^{-1} in 0.25 M GdmCl. The faster rate exhibits roughly linear variation of ln k with respect to [GdmCl], and both phases correspond to a decrease in fluorescence as the protein refolds. This is unexpected, given that GdmCl-unfolded ePRAI has less than half of the native fluorescence at the 325-nm emission wavelength, but fluorescence spectra (excitation wavelength, 278 nm) taken at the same [GdmCl] before unfolding and after refolding are superimposable, confirming that the denatured protein regains native-like tertiary structure soon after dilution into refolding buffer. The observed fluorescence decay behavior indicates the accumulation of at least one fast-forming high-fluorescence intermediate, probably with local structure formation in the vicinity of the tryptophan residues, within the dead time of the experiment (30 ms). The rates and relative changes in fluorescence appear insensitive to the protein concentrations used in kinetics experiments, ruling out the possiblity that either of the kinetic phases corresponds to an aggregation event.

Because of its small amplitude, measurement of relaxation rates for the faster phase is difficult in the transition region (0.75–2.0 M GdmCl). The data suggest, however, that the fast relaxation rates observed in unfolding and refolding are continuous across the region of the equilibrium unfolding transition and may correspond to a single relaxation rate for the system. Overlap of slow-unfolding and slow-refolding rates measured in the equilibrium transition region and the putative C-terminal unfolding region of [GdmCl] indicates that these rates correspond to another single relaxation rate which varies continuously with [GdmCl]. If the two relaxation rates reflect steps in a sequential mechanism for unfolding/refolding, then the slow rate is rate-determining in both the forward and reverse reactions. Observation of only a slow rate for unfolding in 0–1.0 M final [GdmCl] is an indication, however, that this step is dominated by a transition which occurs before the fast relaxation step in unfolding, yet observation of the fast relaxation unfolding phase with a significant decay amplitude at high final [GdmCl] suggests that this step is dominated by a transition which precedes the slow relaxation phase.

This seeming contradiction can be resolved by concluding that the fast and slow relaxation phases at low and high [GdmCl] limits correspond to nonsequential, but kinetically

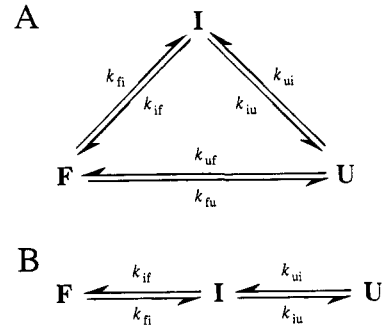

FIGURE 4: Possible mechanisms for ePRAI folding: (A) nonsequential and (B) sequential.

coupled processes (Figure 4A). It is also possible to reconcile the data with a sequential model for folding (Figure 4B), but only if the relaxation rates are assumed to be hybrids of the component rate constants for all GdmCl concentrations in the experimental range. In the nonsequential system one of the relaxation rates approaches $k_{ui} + k_{uf}$ and $k_{fi} + k_{fu}$ under complete refolding and unfolding conditions, respectively, while the other approaches k_{if} and k_{iu} under refolding and unfolding conditions, respectively. In the sequential mechanism, one of the rates approaches k_{ui} and k_{iu} under refolding and unfolding conditions, while the other rate approaches k_{if} and k_{fi} under the respective conditions. A simulated plot of ln k vs [GdmCl] based on the nonsequential model is presented in Figure 3B. Rate constants and m-values for the simulation were chosen arbitrarily to approximate roughly the actual kinetics data in panel A of Figure 3. The simulation models relaxation rates for transitions between three interconverting states, corresponding to the folded, intermediate, and unfolded states in Figure 4. It can be seen that qualitative aspects of the experimental data are present in the simulated curves, in that neither the actual nor the simulated rate constants approach limits expected for sequential three-state folding/unfolding in the 0–5 M GdmCl range. The rate constants used in the simulation contribute to a region of maximum stability for the intermediate after 0.5 M GdmCl, where k_{fi} and k_{if} intersect, followed by a global folding transition centered near the intersection of k_{fu} and k_{uf}, as observed experimentally.

Slow phases in protein refolding have often been attributed to the isomerization of peptide–proline bonds from *cis* to *trans* or vice versa and are typically denaturant-independent (Cook et al., 1979; Kim & Baldwin, 1982; Kiefhaber et al., 1990). ePRAI contains five peptide–proline bonds, all of which are *trans* in the crystal structure. Although we have observed no refolding relaxation rates attributable to peptide–proline isomerizations and have observed restoration of the native fluorescence spectrum within 60 s of manual mixing, it is possible that fluorescence amplitude due to an isomerization phase in refolding is masked by local structure formation in the vicinity of fluorescent side chains or that an isomerization occurs with a rate constant approximately equal to the slow structural refolding rate constant. We have monitored the refolding of ePRAI from 4.4 M urea into 0.4 M urea with and without the presence of 2 μM peptidyl-prolyl isomerase (PPI). This enzyme concentration is sufficient to induce >5-fold increase in the slow-folding rates of the mouse immunoglobulin light chain and the S-protein of RNase A (Lang et al., 1987), but has negligible effect on the refolding of ePRAI.

DISCUSSION

Equilibrium unfolding studies on the $\beta\alpha$-barrel protein ePRAI have indicated the accumulation of a partially folded

intermediate at low [GdmCl] (0–1.0 M), with a loss of 50% of the change in fluorescence and 20% of the far-UV CD amplitude preceding a cooperative global unfolding transition. Stopped-flow experiments indicate the presence of at least one intermediate along the unfolding pathway and more than one along the refolding pathway. The observation of two relaxation rates which vary continuously in refolding and unfolding GdmCl concentrations (0.25–5.0 M) indicates that at least two structural folding processes, as opposed to isomerizations, contribute to the observed kinetic behavior. Detection of the slow-unfolding rate alone at low [GdmCl], with an amplitude significantly larger than observed for this phase at high [GdmCl], suggests that the two processes may be nonsequential and indicates that the slow relaxation rate below 1.5 M GdmCl is primarily due to the transition between the native protein and the intermediate observed under equilibrium conditions. Multistate models for the transitions observed in the folding of ePRAI are presented in Figure 4. Although the data can more easily be reconciled with a nonsequential model for folding (panel A of Figure 4), it is possible the observed intermediates lie on a sequential folding pathway (panel B of Figure 4), which is simply a degenerate case of the nonsequential model. In either case, it is likely that the primary event in refolding is the formation of a high-fluorescence intermediate in the dead time of the experiment. This fast-forming intermediate probably posesses local structure in the vicinity of the two tryptophan residues, and it may have structure similar to that of the molten globule hypothesized as an early folding intermediate for some proteins [see Dobson (1991) for a review].

Given that ePRAI contains only two tryptophan residues (W356 protruding from strand 5 into the barrel core and W391 packed between helices 6 and 7 near the C-terminus), the large fluorescence loss and accompanying CD changes from 0 to 1.0 M GdmCl suggest two salient possibilities as to the structure of the putative equilibrium unfolding intermediate: (1) The intermediate is partially unstructured in the vicinity of both tryptophans, though it retains a majority of native-like secondary structure. (2) The intermediate is unfolded primarily in the vicinity of one of the tryptophans while local structure in the vicinity of the other remains largely unaffected. The fact that W356 is packed well into ePRAI's hydrophobic core suggests that local unfolding in this region would be difficult without significant disruption to the overall structure of the molecule and concomittant large changes in the far-UV CD spectrum, which are not observed. The hypothesis that the structural intermediate is locally unfolded only in the vicinity of the C-terminal W391 is thus more consistent with our data.

This hypothesis is also supported by experimental evidence from other laboratories. Most pertinent are fragment studies on derivatives of yPRAI, the closely related *Saccharomyces cerevisiae* homologue of ePRAI, which demonstrated compact folding for a C-terminally truncated fragment lacking the final two $\beta\alpha$ secondary structural units (Eder & Kirschner, 1992). The N-terminal fragment underwent a cooperative unfolding transition when titrated with GdmCl, while a C-terminal fragment containing only the final two $\beta\alpha$-units denatured uncooperatively but associated with the large N-terminal fragment to generate a correctly folded and enzymatically active complex (Eder & Kirschner, 1992). According to crystallographic data, the C-terminal portion of ePRAI contains relatively low amounts of secondary structure, suggesting that this region may be dynamically unstable even in 0 M GdmCl (Wilmanns, 1991). Matthews and co-workers have extensively characterized a C-terminally unfolded intermediate in the unfolding of the α-subunit of tryptophan synthase, a related protein in the $\beta\alpha$-barrel superfamily (Miles et al., 1982; Beasty & Matthews, 1985; Tsuji et al., 1993), and it might be reasonable to expect such an intermediate in other $\beta\alpha$-barrel proteins. Our data, combined with the other results, support but do not prove the reasonable hypothesis that the ePRAI equilibrium unfolding intermediate is C-terminally unfolded in such a way as to expose W391 to fluorescence quenching by solvent but not to result in much loss of secondary structure.

The consistency of preliminary data on the folding of ePRAI with data on the folding of the tryptophan synthase α-subunit again raises the question of whether the members of this family of proteins share a common folding mechanism. The folding of a third $\beta\alpha$-barrel protein, a monomeric aldolase from *Staphylococcus aureus*, has been studied by Rudolph et al. (1992). Using manual mixing techniques, the authors observe a relaxation rate whose magnitude and GdmCl dependence are similar to those of the slower relaxation rate in ePRAI folding kinetics. The GdmCl dependence of a relaxation rate constant reflects changes in solvent-accessible area along the course of the forward and reverse reaction pathways (Pace, 1986) and is thus indicative of the structural nature of the folding process. Although Rudolph et al. (1992) claim that the aldolase relaxation rate may arise from coupling between a structural kinetic relaxation and the isomerization of a peptide–proline bond with a similar rate constant, they also note that double-jump experiments fail to support this hypothesis. Further, the presence of a fast-refolding phase within the dead time of their experiment suggests that the slower rate may correspond to decay from an intermediate to the native conformation. This evidence for similarity between the folding pathways of ePRAI and staphylococcal aldolase is merely circumstantial, however, and provides only speculative support for a common folding mechanism between these two proteins.

Previous analyses have used evidence of a shared folding pathway as support for an evolutionary relationship between PRAI and tryptophan synthase (Eder & Kirschner, 1992; Tsuji et al., 1993). Despite the lack of significant sequence homology, there are several arguments for such a relationship between these two enzymes on the tryptophan biosynthesis pathway (Wilmanns et al., 1991), and a study of homologous RNases from a variety of sources has demonstrated some evolutionarily related conservation of folding pathway despite differences in sequence, proline content, and posttranslational modification (Krebs et al., 1983). But just as structural surveys of the $\beta\alpha$-barrel family have implicated $(\beta\alpha)_8$ packing as an optimal solution to the problems of hydrophobic core packing and β-strand organization in closed manifolds (Lasters et al., 1988; Lesk et al., 1989), it is not unlikely that the mechanism of domain folding through a C-terminally unstructured intermediate has its roots in structural and kinetic considerations common to and dictated by the $(\beta\alpha)_8$ tertiary fold. Experiments which demonstrate that circularly permuted analogues of yPRAI with shared native-like tertiary structure have similar kinetic and equilibrium unfolding behaviors (Luger et al., 1989) support the hypothesis that $\beta\alpha$-barrel folding is intrinsic to its global fold and not specific to the sequence or evolution of a given protein.

The folding of $\beta\alpha$-barrels represents an extreme case of folding which can be decomposed into fast local events (formation and association of adjacent $\beta\alpha$-units) and a slower nonlocal event ("closing" of the barrel by association of the

N- and C-terminal ends of the protein). This nonlocal event may be limiting because of the steric difficulty of fitting the ends of the protein together in a perfect barrel and because of the entropy loss upon restraining the two free termini. These considerations possibly favor the formation of smaller compact intermediates with lower stability but less stringent packing requirements, making them kinetically accessable to a folding protein. One of the attractive features of the nonsequential folding mechanism presented in Figure 4A is that it allows for a statistical distribution between folding molecules which find the correct nonlocal interactions quickly and fold directly to the native state and those which "accidentally" collapse into a non-native closed conformation and must subsequently rearrange to the native structure.

Based on lattice model Monte Carlo simulations of tryptophan synthase, Godzik et al. (1992) have predicted a loose six-stranded $\beta\alpha$-barrel as an N-terminal folding intermediate. They suggest that melting of such a structure may be the rate-limiting step in barrel folding, which would be consistent with the observed GdmCl independence of the slow-refolding relaxation rate for ePRAI if this relaxation corresponds to a rate constant for refolding from the intermediate. N- and C-terminal six-stranded folding intermediates were also predicted from simulations of TIM, which is less likely to share a common ancestor with PRAI (Farber & Petsko, 1990). Lasters et al. (1988) have suggested that six-stranded $\beta\alpha$-barrels can be accommodated under certain combinations of geometric packing parameters, which may in part account for the relative stability of a six-$\beta\alpha$-unit folding intermediate.

It is possible that other properties of $\beta\alpha$-barrel packing, for instance, the propensity of barrels to begin with an N-terminal β-strand, dictate a tendency for formation of N-terminal kinetic and equilibrium intermediates. Two distinct hydrophobic packing architectures have been found in $\beta\alpha$-barrels (Lesk et al., 1989). Both ePRAI and the tryptophan synthase fall into the class of $\beta\alpha$-barrels in which the first β-strand contributes two residues to the barrel core, raising the possibility that this hydrophobic architecture correlates with the presence of an N-terminal folding intermediate. Because of the relative dearth of information on the denaturation of $\beta\alpha$-barrel proteins, it is certainly too early to conclude that a specific common folding mechanism holds. Furthermore, it should be emphasized that data on the folding of ePRAI is still preliminary; precise characterization of the ePRAI structural folding intermediate awaits investigation by nuclear magnetic resonance and site-directed mutagenesis studies, and it cannot yet be concluded that the ePRAI folding intermediate has the same structure as the C-terminally unfolded intermediate observed in the folding of tryptophan synthase. However, new experimental evidence, though as yet incomplete, for similarity between the folding pathways of at least two $\beta\alpha$-barrel enzymes with virtually no sequence homology does fuel further speculation about the relationship between the native three-dimentional structure and the folding pathway for these enzymes and for proteins in general.

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