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Stable Substructures of Eightfold $\beta\alpha$-Barrel Proteins: Fragment Complementation of Phosphoribosylanthranilate Isomerase†

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ABSTRACT: The $(\beta\alpha)_8$ (or "TIM")-barrel protein phosphoribosylanthranilate isomerase from *Saccharomyces cerevisiae* was cleaved between the sixth and seventh $\beta\alpha$ module to test the capacity of the resulting fragments to adopt native format autonomously. The fragments, which were expressed from separate coding sequences, were soluble and monomeric. The amino-terminal fragment p_1 was compact, possessed an almost nativelike far-UV but a strongly reduced near-UV CD spectrum, and unfolded cooperatively with guanidinium chloride. In contrast, the carboxyl-terminal fragment p_2 was less compact than fragment p_1, possessed only a weak far-UV and no detectable near-UV CD spectrum, and unfolded noncooperatively. The fragments assembled stoichiometrically to a complex with $K_d = 0.2$ μM, which was enzymically almost fully active. The rate of assembly was limited by a first-order process, probably the isomerization of the carboxyl-terminal fragment p_2 to an assembly-competent structure. These results support a folding mechanism that comprises an intermediate with the first six $\beta\alpha$ units folded in roughly native format and the last two $\beta\alpha$ units partially unfolded. The similar behavior of the analogous fragments of the α subunit of tryptophan synthase supports the hypothesis that these two $(\beta\alpha)_8$-barrel proteins have evolved from a common ancestor.

Denatured proteins refold in vitro by collapsing to a compact state that subsequently rearranges itself to the native structure. This "molten globule" (Kuwajima, 1989) or "collapsed intermediate" (Kim & Baldwin, 1990) state can possess a circular dichroism spectrum in the peptide absorbance region that suggests a content of secondary structure similar to that of the stable folded state. Nativelike secondary and supersecondary structures of segments of the polypeptide chain could

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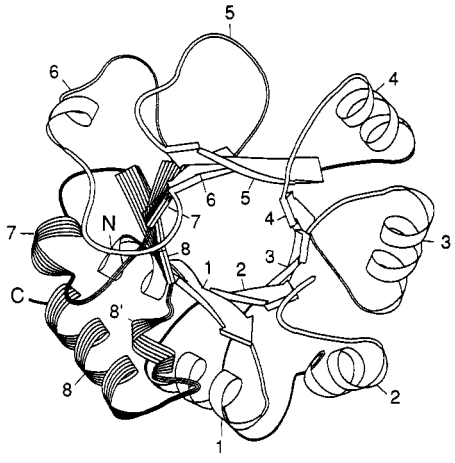

FIGURE 1: Schematic structure of yeast phosphoribosylanthranilate isomerase as an 8-fold $\beta\alpha$-barrel enzyme. Ribbon drawing of the model (Eder & Wilmanns, 1992). View down the barrel axis with the active site at the carboxy terminus of the central β barrel facing up. (N) Amino terminus; (C) carboxy terminus. β strands and α helices are numbered consecutively from the amino terminus. The yeast enzyme may have an extra helix, α_0, at its amino terminus. The region that corresponds to fragment p_2, which comprises $\beta_7\alpha_7\beta_8\alpha_8'$ and α_8, is hatched. Fragment p_1 corresponds to the region from α_0 to α_6.

play an important role in directing the folding process. However, such "folding units" (Baldwin, 1989) cannot be identified unambiguously by merely inspecting the structure of the protein.

An important approach to study the role of domains or subdomains in protein folding is to prepare fragments of the polypeptide chain (Wetlaufer, 1981; Taniuchi et al., 1986). If a given fragment folds to its native format by itself, this independent behavior tentatively identifies a folding unit. Moreover, contiguous or overlapping fragments can assemble to active complexes. Thus, by artificially converting protein folding from a (first-order) isomerization to a (second-order) assembly process, information could possibly be obtained on how the different substructures of the protein interact during the folding process (Taniuchi et al., 1977; Labhardt & Baldwin, 1979a,b; Hall & Frieden, 1989).

Phosphoribosylanthranilate isomerase (PRAI)[1] from *Escherichia coli* (ePRAI) has the $(\beta\alpha)_8$ (or "TIM")-barrel structure (Priestle et al., 1987; Wilmanns et al., 1992) that is considered to be a single domain. It is a member of the predominant protein fold found in at least 19 other enzymes (Farber & Petsko, 1990; Rouvinen et al., 1990; Wilson et al., 1991), and therefore an interesting model for protein folding studies.

Native ePRAI is fused to the carboxy terminus of indoleglycerolphosphate synthase, another $(\beta\alpha)_8$ protein on the pathway of tryptophan biosynthesis. By contrast, PRAI from *Saccharomyces cerevisiae* (yPRAI) is naturally a monomeric protein (Braus et al., 1988). Its sequence shares 28% identical and 46% equivalent residues with ePRAI, but its structure is unknown. However, its sequence has been fitted to the coordinates of ePRAI to generate a model in which the secondary structural elements and the conserved residues occupy equivalent positions (Figure 1; Eder & Wilmanns, 1992). Significant deviations are found at the extended amino terminus of yPRAI, and in the loops between α-helix 2 and β-strand 3 as well as between α-helix 6 and β-strand 7. Here yPRAI has insertions of nine and seven residues, respectively.

The carboxyl-terminal region of yPRAI from β_7 to α_8 has been transposed to the amino terminus of the protein to generate a circularly permuted variant of yPRAI that is fully active (Luger et al., 1989). Moreover, in the case of ePRAI, the same region is responsible for binding the substrate's phosphate moiety (Wilmanns et al., 1991). A short α helix (α_8'), which is located in the loop between the canonical β_8 and α_8 elements, actually stabilizes the bound phosphate moiety of the substrate with its helix dipole. In addition, the same substructure with the same function and with significant tertiary and sequence identity has been found in both indoleglycerolphosphate synthase and the α subunit of tryptophan synthase. Thus, these three enzymes, which catalyze sequential steps in the biosynthesis of tryptophan, seem to be related by divergent evolution.

The unfolding transition of the α subunit of tryptophan synthase induced by guanidinium chloride (GuCl) at equilibrium is biphasic (Yutani et al., 1980). Limited proteolysis generates two contiguous fragments (α-1 and α-2; Miles et al., 1982) that can assemble to an active complex. The small fragment comprises the secondary structural elements $\alpha_6\beta_7\alpha_7\beta_8\alpha_8$, again including a short α helix (α_8') in the loop between β_8 and α_8. At equilibrium and in the presence of 1.2 M GuCl, the α subunit of tryptophan synthase exists as a stable intermediate, in which the region corresponding to the fragment α-2 is unfolded, whereas the region corresponding to α-1 remains folded. Since ePRAI and the α subunit of tryptophan synthase appear to be evolutionarily related, we wished to determine whether the analogous fragments of yPRAI have properties resembling those of α-1 and α-2.

In this work, we have dissected the polypeptide chain of yPRAI into a large amino-terminal fragment, p_1 (β_1–α_6), and a small carboxyl-terminal fragment, p_2 (β_7–α_8; cf. Figure 1), at the level of the TRP 1 gene (Tschümper & Carbon, 1980). Fragment p_1 possesses a defined structure and can associate stoichiometrically with fragment p_2 to form a fully active complex. Thus, the folding mechanism seems to have been conserved during divergent evolution of PRAI and the α subunit of tryptophan synthase.

Experimental Procedures

Materials. Restriction enzymes and other DNA-modification enzymes were purchased from Boehringer Mannheim Corp., Pharmacia, or United States Biochemical Corp. The recently available ultrapure factor Xa was from Boehringer Mannheim Corp. and proved to be superior to all other preparations. Antiserum versus yPRAI for immunoblotting was kindly provided by M. Eberhard. Ultrapure GuCl, which was used for spectroscopic studies, was from Schwarz/Mann. All other reagents were of analytical grade.

Buffers. Buffer A was 0.1 M potassium phosphate at pH 7.5, containing 5 mM DTE and 2 mM EDTA. Buffer B consisted of 0.05 M potassium phosphate at pH 7.8, containing 0.1 M KCl, 2 mM DTE, 1 mM EDTA, and 10% glycerol.

Plasmids and Strains. The vector pDS78/RBSII DHFR was kindly provided by D. Stüber. The vector pDS56/RBSII-1 (Certa et al., 1986) with the *E. coli* strain SG200-50 (strain collection D. Stüber) was used for heterologous gene expression, as described earlier (Luger et al., 1989). SG200-50

[1] Abbreviations: CD, circular dichroism; DEAE, diethylaminoethyl; DTE, dithioerythritol; EDTA, (ethylenedinitrilo)tetraacetic acid; GuCl, guanidinium chloride; mDHFR, mouse dihydrofolate reductase; PRAI, phosphoribosylanthranilate isomerase; yPRAI, PRAI from *Saccharomyces cerevisiae*; xPRAI, extended recombinant version of yPRAI; xPRAI*, variant of xPRAI with additional *BglII* restriction site in coding sequence; ePRAI, PRAI domain of the bifunctional enzyme indoleglycerolphosphate synthase:PRAI from *Escherichia coli*; p_1, amino-terminal fragment of xPRAI* ranging from β_1 to α_6; p_2, carboxyl-terminal fragment of xPRAI* ranging from β_7 to α_8; SDS, sodium dodecyl sulfate.

is a *lonA*⁻ (*Tn5*) derivative of strain MC 4110 (*F*⁻, Δ *LacU169*, *araD139*, *rpsL*, *relA*, *thiA*, *flbB*) (Casabadan, 1976).

Genetic Engineering Methods. Double-stranded plasmid DNA was isolated according to Humphreys et al. (1975; modified version for large-scale preparation) and Birnboim and Doly (1979; small scale). Two complementary oligonucleotides (5′-GATCCCGGGGTTATCGAAGGTCGTATCGAG-3′ and 5′-GATCCTCGATACGACCTTCGATAACCCCGG-3′) were synthesized on an Applied Biosystems ABI 380B oligonucleotide synthesizer, and purified separately by gel filtration on Sephadex G-25.

Ligated nucleotide regions were confirmed by checking the isolated plasmid DNA for the loss of the relevant restriction sites and by sequencing the variant genes (Sanger et al., 1977). All other genetic engineering methods followed standard protocols (Sambrook et al., 1989).

Expression and Purification of Proteins. Cells harboring the corresponding expression plasmids were cultivated and induced as described previously (Luger et al., 1989). All purification steps were performed at 4 °C, unless stated otherwise. For the purification of the fragment p_1 from inclusion bodies, 7.5 g of wet cells was first washed with buffer A containing 0.3 mM phenylmethanesulfonyl fluoride and then resuspended in 15 mL of the same buffer. Cells were ruptured by sonication at 0 °C. After centrifugation of the homogenate at 16500*g* for 40 min, the pellet was dissolved in buffer A containing 4 M GuCl at room temperature for 45 min. The solution was centrifuged (16500*g* for 30 min), and the clear supernatant was loaded onto a column of Sephacryl S-300 (85 cm × 5 cm) equilibrated with 3 M GuCl in 0.05 M potassium phosphate buffer at pH 7.5, 2 mM DTE, and 1 mM EDTA. Fractions that contained the fragment p_1 as checked by polyacrylamide gel electrophoresis in the presence of SDS as well as immunoblotting were pooled, diluted 3-fold with the column buffer, and dialyzed exhaustively against buffer B. The protein solution was dripped into liquid nitrogen and stored at −70 °C.

The DHFR/Xa/p_2 fusion protein was purified from inclusion bodies as described previously for mutants of yPRAI (Luger et al., 1989) with the exception that DEAE-Sepharose-Cl 6B was used instead of DEAE-Sepharose fast-flow for anion-exchange chromatography, because the fusion protein did not bind to the latter column material. The fractions that had DHFR activity (Baccanari et al., 1975) were pooled, dripped into liquid nitrogen, and stored at −70 °C. The average yields (mg of protein/g of wet cell paste) of various gene products were as follows: xPRAI, 8.6; p_1, 0.4; DHFR/Xa/p_2, 10.4; p_2, 2.1.

Cleavage of DHFR/Xa/p_2 with Factor Xa and Isolation of Fragment p_2. The DHFR/Xa/p_2 fusion protein was dialyzed against the cleavage buffer for factor Xa (0.05 M Tris-HCl buffer at pH 8.0, containing 0.1 M NaCl and 1 mM $CaCl_2$, following the supplier's specifications) and concentrated by ultrafiltration with Amicon PM 10 filters to a concentration of about 1.3 mg/mL. Factor Xa (1 mg/mL in the same buffer) was added to 1% of the fusion protein by weight. Incubation was carried out on ice for 40 h, since low temperature prevented aggregation of the fusion protein. The protein solution was then heated for 3 min at 65 °C to precipitate both mDHFR and factor Xa, cooled to 4 °C, and centrifuged. The supernatant, which contained the fragment p_2 in pure form, was stored at −70 °C.

Determination of Protein Concentration. The molar extinction coefficient at 280 nm of xPRAI* was determined by second-derivative absorbance spectroscopy (Levine & Federici, 1982) to be 32200 M^{-1} cm^{-1}, using a Hewlett-Packard 8452A diode array spectrophotometer with 1-nm resolution. Because four out of five tyrosines and all three tryptophan residues of xPRAI* are located in the p_1 region, the ϵ_{280} for this fragment was calculated to be 43100 M^{-1} cm^{-1}. The calculated extinction coefficient of fragment p_2 at 280 nm is impractically small (1100 M^{-1} cm^{-1}) because it contains only a single tyrosine residue (Y_{225}, cf. Figure 2). Therefore, the concentration of fragment p_2 was estimated by amino acid analysis. The analysis was performed after hydrolysis in 6 N HCl for 24 h at 110 °C followed by dabsylation and quantitative reverse-phase high-performance liquid chromatography on a C-18 column (Merck) as described by Knecht and Chang (1986).

Determination of Molecular Weight. The apparent molecular weights of the folded fragments were determined by gel filtration on a column of Superose 12 (1 cm × 30 cm), using a Merck-Hitachi high-performance liquid chromatography system. The column was equilibrated and eluted with buffer B at 22 ± 2 °C. After injection of 20-μL aliquots of protein solution, the elution profile was monitored by the absorption at 230 nm. The column was calibrated with proteins of known molecular weight: cytochrome *c* (12400); horse heart myoglobin (17000); bovine carbonic anhydrase (30000); ovalbumin (43000); bovine serum albumin (66300); beef heart lactate dehydrogenase (140000); and rabbit muscle phosphorylase (194000).

Polyacrylamide gel electrophoresis in the presence of SDS was conducted according to Lämmli (1970) and Studier (1973) using gels containing 12.5% acrylamide. The following proteins were used as molecular weight markers: bovine trypsin inhibitor (6500); cytochrome *c* (12400); horse heart myoglobin (17000); the α subunit of tryptophan synthase (28700); glyceraldehyde-3-phosphate dehydrogenase (35700); ovalbumin (43000); bovine serum albumin (66300); rabbit muscle phosphorylase (97400). In order to detect small protein fragments, the electrophoresis method described by Schägger and Jagow (1987) was followed, using gels containing 10% acrylamide.

Amino-Terminal Sequencing. Amino-terminal sequences were determined by pulsed liquid-phase sequencing on an Applied Biosystems 477A sequencer according to the manufacturer's specifications. Identification of the corresponding phenylhydantoins was carried out on-line on an 120A amino acid analyzer from Applied Biosystems.

Reconstitution of the Active Complex from Fragments. Fragment p_1 (5–10 μM in buffer B) was mixed with a stoichiometric concentration of fragment p_2 and incubated for 30 min at 4 °C. For titration experiments, fragment p_1 (5.8 μM in buffer B) was mixed with a series of aliquots of fragment p_2 (0.285 mM in buffer B). After the mixture had been incubated for 30 min at 4 °C, PRAI activity was measured at 15 °C according to Hommel et al. (1989) in 50 mM Tris-HCl buffer, pH 8.0. Progress curves were linear for at least 1 min, showing that the complex was stable under assay conditions.

Association kinetics were measured in buffer B at 15 °C. After the fragments were mixed manually, time-dependent formation of active complex was determined by removing aliquots for PRAI activity assays. To check the dependence of the association on the concentration of the fragments, a stock solution of fragment p_1 (final concentration 7 μM) was mixed with different molar ratios of fragment p_2.

The steady-state kinetic parameters were determined by analyzing entire progress curves according to Duggleby and Morrison (1977); titration data were fitted by means of a nonlinear least-squares fit procedure (Eberhard, 1990).

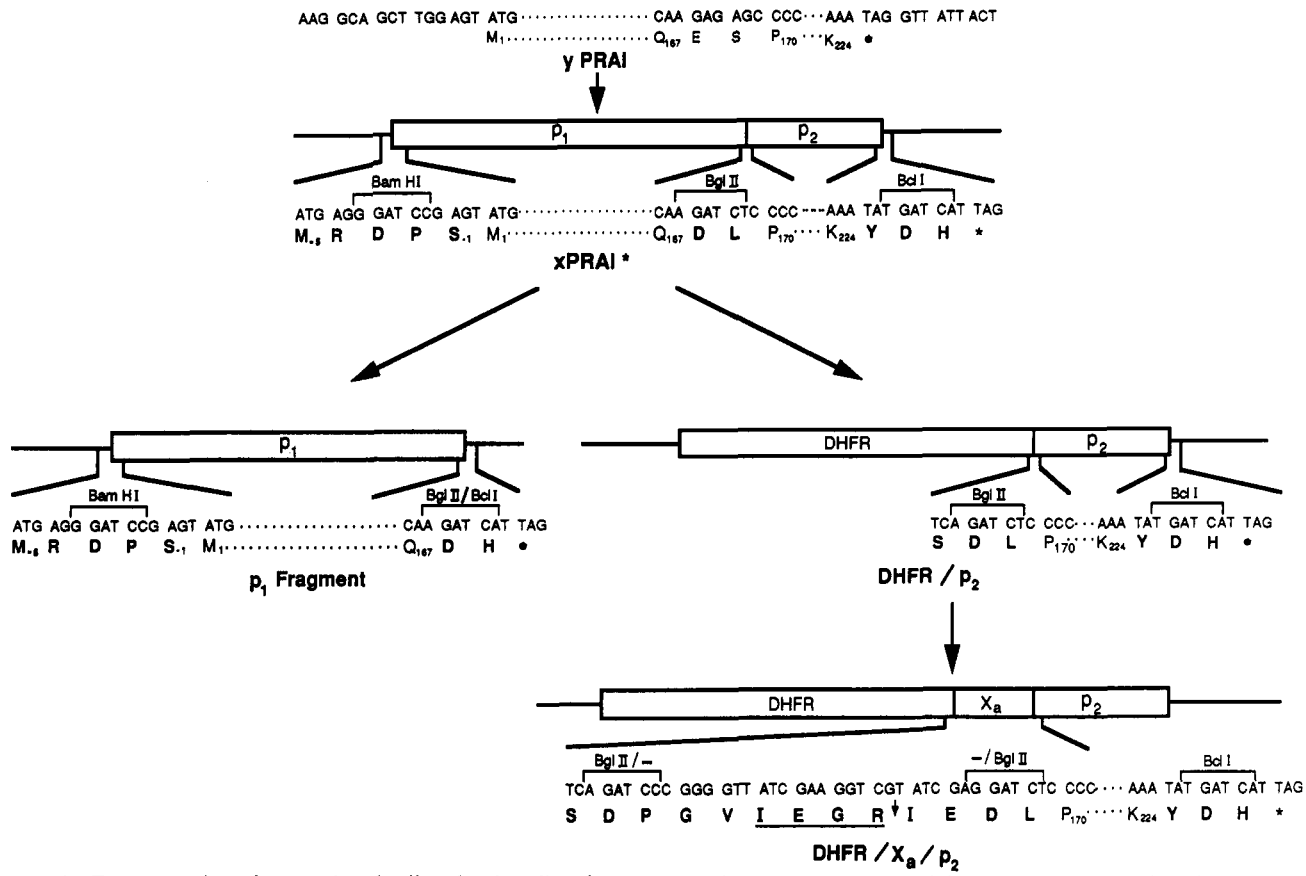

FIGURE 2: Fragmentation of yeast phosphoribosylanthranilate isomerase at the genetic level. Partial amino acid and nucleotide sequences. New amino acids are shown in boldface type; new and modified restriction sites are indicated. (yPRAI) Wild-type phosphoribosylanthranilate isomerase from yeast and the TRP I gene (Tschümper & Carbon, 1980). (xPRAI*) Extended wild-type protein with additional *Bam*HI, *Bgl*II, and *Bcl*I restriction sites. (Fragment p_1) Amino-terminal fragment; cf. Figure 1. (DHFR/p_2) Fusion protein of mouse DHFR and the last two $\beta\alpha$ units of xPRAI*. (DHFR/Xa/p_2) Fusion protein with linker for factor Xa cleavage. A synthetic oligonucleotide linker was introduced into the *Bgl*II site of DHFR/p_2. The specific factor Xa recognition site is underlined, and the site of cleavage by factor Xa is indicated by the arrow.

Circular Dichroism Measurements. CD measurements were carried out with a Cary Model 60 instrument at 15 °C. Far-UV CD (250–200 nm) and near-UV CD (320–250 nm) spectra were measured at a protein concentration of 5–10 μM in 0.1- and 5-cm cells, respectively. Mean residue ellipticity values ($[\theta]_{mrw}$) were calculated by using the expression:

$$[\theta]_{mrw} = 100\theta_{obs}/lc \text{ (deg cm}^2 \text{ dmol}^{-1})$$

where θ_{obs} is the observed ellipticity in degrees, c is the molar residue concentration, and l is the light path in centimeters (Schmid, 1989). The mean residue molecular weights were calculated from the amino acid sequences to be 108.3 for PRAI, 109.6 for the p_1, and 105.1 for the p_2 fragment.

For equilibrium unfolding experiments, the ellipticity at 222 nm was measured on protein solutions of 0.1–0.05 mg/mL in a cell having a 0.2-cm light path. Measurements were made after incubating the proteins for 3 h at 15 °C in buffer B with the indicated final concentrations of GuCl. After 2 h, no further change in the CD signal was obtained. Refolding experiments were performed by first unfolding the protein at a concentration of 2 mg/mL in buffer B containing 5 M GuCl for 2 h at 15 °C. Then the solution was diluted with buffer B to the desired final concentration of GuCl, and CD spectra were measured after further 3 h at 15 °C.

Fluorescence Measurements. Uncorrected fluorescence emission spectra were measured with an SLM 8000 single photon counting recording fluorometer at 15 °C with excitation at 280 nm. The slit-width of both monochromators was 8 nm. Protein concentrations were 3.4 μM in buffer B.

RESULTS

Choice of Cleavage Site. The cleavage site of yPRAI was chosen in the loop between α-helix 6 and β-strand 7 rather than between β-strand 6 and α-helix 6 (the loop of the α subunit of tryptophan synthase that is cleaved by trypsin; Miles et al., 1982), for the following reasons: (1) the transposition of the sequence that corresponds to fragment p_2 to the amino terminus of fragment p_1 led to a fully active circularly permuted variant (Luger et al., 1989). (2) The p_2 region comprises the evolutionarily conserved phosphate binding subdomain of PRA isomerase and indoleglycerolphosphate synthase as well as the α subunit of tryptophan synthase (Wilmanns et al., 1991). (3) The cut between α-helix 6 and β-strand 7 is on the face of the barrel opposite to the active site. Thus, correct fragment assembly should lead to an enzymically active fragment complex.

Constructions. The construction of the coding sequences of the two fragments is shown schematically in Figure 2. xPRAI is an extended variant of yPRAI with five additional residues added to the amino terminus (Luger et al., 1990). In contrast, the gene of xPRAI* has an additional *Bgl*II restriction site in the region coding for the loop that connects α-helix 6 and β-strand 7. The protein xPRAI* has the same steady-state enzyme kinetic constants and unfolding transitions as yPRAI and was therefore used as reference "wild-type" protein for all measurements.

The restriction sites available in the gene of xPRAI* had the same protruding ends and were positioned in such a way that religated nucleotide regions maintained the overall reading

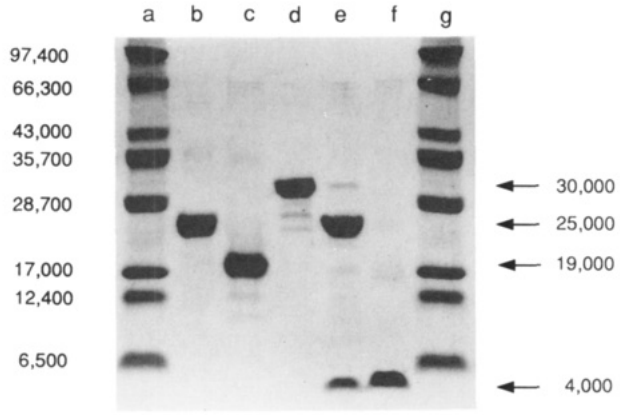

FIGURE 3: Purity of fragments p_1 and p_2. Polyacrylamide gel electrophoresis in presence of SDS. Lanes a and g, molecular weight marker proteins with associated M_r values on the left; arrows on the right margin indicate the interpolated M_r values of the various protein constructs. Lane b, xPRAI*; lane c, purified fragment p_1; lane d, purified DHFR/Xa/p_2 fusion protein; lane e, products of fusion protein of lane d after cleavage by factor Xa; lane f, isolated fragment p_2.

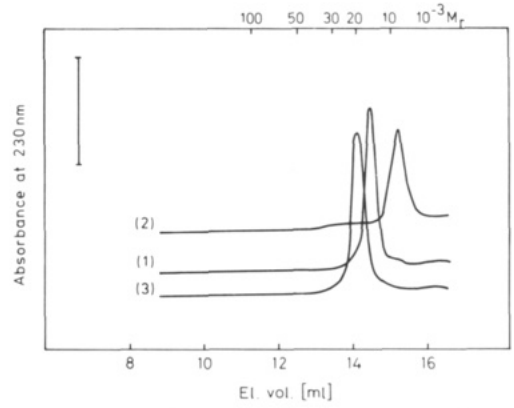

FIGURE 4: Fragments of xPRAI* are monomeric and form a stoichiometric complex. Gel filtration under native conditions. Chromatography in buffer B at 22 °C. (1) Fragment p_1; (2) fragment p_2; (3) the equimolar mixture of both fragments. The flow rate was 0.4 mL/min. Elution profiles monitored by the absorption at 230 nm after injection of protein samples of 0.1 mg/mL. The bar represents 0.01 absorbance unit; d = 0.5 cm. 10^{-3} M_r values on upper ordinate from calibration with marker proteins.

frame. Deletion of the region coding for fragment p_2 and insertion of a stop codon led to the coding sequence of fragment p_1 with two extra amino acid residues (Asp and His) at its carboxyl terminus.

Initial experiments with plasmids capable of expressing fragment p_2 either by itself or in combination with fragment p_1 (having a suitable intercistronic region between both coding sequences; K. Luger, unpublished work) were not successful. The crude extracts contained none of the expected protein bands as detected by polyacrylamide gel electrophoresis in presence of SDS, and either silver staining or immunoblotting. Furthermore, the use of the T_7-expression system (Studier et al., 1990) also failed to produce fragment p_2.

As an alternative route, we constructed a fusion protein by replacing the sequence encoding fragment p_1 by the gene of mouse dihydrofolate reductase (mDHFR; Bujard et al., 1987), which led to the intermediate DHFR/p_2. The preserved *Bgl*II site was used to insert a cassette coding for a factor Xa cleavage site. This short double-stranded DNA fragment was assembled by hybridizing two synthetic 30mer oligonucleotides, designed (1) to code for a flexible loop between mDHFR and fragment p_2 that carries a cleavage site for factor Xa as close as possible to the beginning of p_2, (2) to give 5′ ends compatible with *Bgl*II, (3) to eliminate the *Bgl*II site at both ends after insertion into the vector, and (4) to contain a stop codon for the wrong orientation of the double-stranded linker. The two latter conditions facilitated the subsequent screening for mutants. Transformants resistant toward ampicillin were screened for loss of the unique *Bgl*II site and for production of full-length protein. About 50% of colonies carried the correct orientation.

The inserted decapeptide contains the factor Xa recognition site Ile-Glu-Gly-Arg-↓ [the arrow in Figure 2 shows the site of cleavage (Nagai & Thogersen, 1987)]. As in the native substrate prothrombin, an Ile residue was introduced carboxyl terminal to the cleavage site. Thus, digestion with factor Xa should generate fragment p_2 with two additional amino acid residues, Ile and Glu, at its amino terminus (cf. Figure 2).

Expression and Purification of the Fragments. The fragment p_1 and the DHFR/Xa/p_2 fusion protein were expressed separately in *E. coli*, and the major fraction of each protein was insoluble. Each protein was solubilized, refolded, and purified to about 95% purity as judged by polyacrylamide gel electrophoresis in the presence of SDS (Figure 3). The fragment p_1 precipitated at concentrations above 0.1 mg of protein/mL, but aggregation was suppressed by low temperature and adding both 0.1 M potassium chloride and 10% glycerol to buffer B. Moreover, the pH was crucial, having to be above 7.6. Addition of other salts such as ammonium sulfate (up to a concentration of 1 M) or potassium phosphate or potassium chloride (0.5 M and 1 M, respectively) did not increase its solubility and had no effect on the CD spectrum of fragment p_1.

Because the fusion protein is also prone to aggregation, it was treated with factor Xa at low temperature. No unspecific cleavage was observed even at incubation times of more than 90 h at 0 °C. After cleavage, fragment p_2 was isolated by heating the protein solution briefly. In contrast to mDHFR and factor Xa, fragment p_2 did not precipitate upon heating, even at concentrations of 5 mg/mL. The observed amino acid compositions of the fragments were in good agreement with the calculated values (data not shown). The expected amino-terminal sequences of p_1 (MRDPSMSVINFTGSS...; cf. Figure 2) and of p_2 (IEDLPESLHFMLAGG...) were confirmed by amino-terminal sequencing.

Properties of the Fragments. (*a*) *The Fragments Are Monomers.* Analysis by gel electrophoresis in the presence of SDS showed good agreement with the calculated M_r 18 789 of fragment p_1 but led to a somewhat lower estimate (4000) than the calculated M_r of 6096 of fragment p_2 (Figure 3). It is known that the molecular weights of small peptides cannot be determined reliably by this method (Swank & Munkres, 1971). The same problem was encountered with the α-2 fragment of the α subunit of tryptophan synthase, where the M_r was found to be 3600 instead of 8300 (Higgins et al., 1979).

Gel filtration under native conditions indicated that the fragments were monomeric (Figure 4). The apparent M_r of fragment p_1 by this method was calculated to be 17 500. It must therefore be folded compactly. The apparent M_r of fragment p_2 was 8000. By comparison to the known M_r (6096), the Stokes radius of this fragment is larger than expected for a compactly folded polypeptide chain.

(*b*) *Circular Dichroism and Fluorescence Spectroscopy.* Circular dichroism spectra were measured of the fragments and their stoichiometric complex as a relative indicator of differences in secondary and tertiary structure (Bayley, 1980; Schmid, 1989). Figure 5A shows the far-UV CD spectra of the proteins under native conditions. Limited sensitivity of

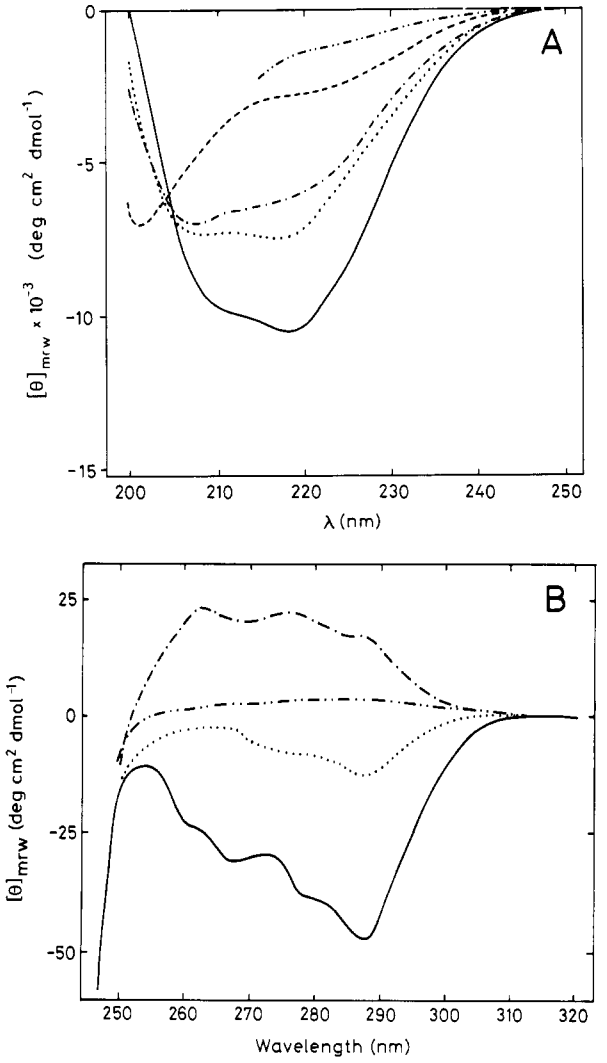

FIGURE 5: CD spectra of xPRAI*, its fragments, and the fragment complex. Protein concentrations were 5–10 μM in buffer B at 15 °C. (A) Far-UV CD spectra measured in a cell with 0.1-cm path length. (—) xPRAI*; (-·-) fragment p_1; (---) fragment p_2; (···) fragment complex; (-··-) superimposed spectra of xPRAI* and both fragments in 4 M GuCl. (B) Near-UV CD spectra recorded in a cell with 5-cm light path. (—) xPRAI*; (-·-) fragment p_1; (···) fragment complex; (-··-) xPRAI* in 4 M GuCl.

the instrument unfortunately prevented measurements below 200 nm. The spectrum of the p_1/p_2 fragment complex is qualitatively similar to that of xPRAI*, but has only 75% of its amplitude at 218 nm. Whereas the spectrum of fragment p_1 is only slightly different from that of the complex, the spectrum of fragment p_2 has a much smaller amplitude and a different shape. However, even xPRAI* denatured by adding GuCl appears to have some residual secondary structure (Yang et al., 1986).

As shown in Figure 5B, the near-UV CD spectra of xPRAI* and fragment p1, which are determined by aromatic amino acids and their environment in the protein, differ qualitatively. Whereas the amplitude is negative for xPRAI*, it is positive for fragment p_1. By contrast, fragment p_2 has only negligible amplitude in the aromatic CD region (data not shown), because it contains only one Tyr residue at the artifically extended carboxyl terminus (cf. Figure 2), and no Phe or Trp residues.

Fluorescence emission spectra of tryptophan residues in the isolated fragments and the fragment complex were used to monitor qualitatively any differences in the polarity of their environment (Schmid, 1989) relative to that of xPRAI*. All

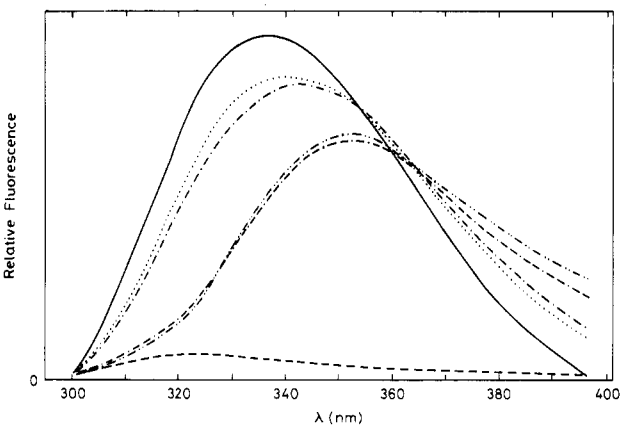

FIGURE 6: Fluorescence emission spectra of xPRAI*, its fragments, and the fragment complex. Fluorescence was excited at 280 nm. Protein concentrations were 3.4 μM in buffer B. Temperature, 15 °C. (—) xPRAI*, 0 M GuCl; (-·-) fragment p_1, 0 M GuCl; (---) fragment p_2, 0 M GuCl; (···) fragment complex, 0 M GuCl; (-··-) xPRAI*, 6 M GuCl; (-···-) fragment p_1, 6 M GuCl.

three tryptophan residues of yPRAI are located in fragment p_1. According to the model (cf. Figure 1), W105 is near the amino terminus of α-helix 4 (buried between α-helix 4 and β-strand 5), W157 is near the amino terminus of α-helix 6 (buried between α-helix 6 and β-strand 7), and W163 is the fourth residue out of 14 that comprise the loop connecting α-helix 6 and β-strand 7. W163 is close to the carboxyl terminus of fragment p_1.

Figure 6 shows that the fluorescence emission spectrum of the fragment complex is shifted by about 5 nm to the red by comparison to the spectrum of xPRAI*. It is also quenched by approximately 10%. The spectrum of fragment p_1 is almost identical to that of the fragment complex. In contrast, the single Tyr residue of fragment p_2 fluoresces at 320 nm, but only weakly. Unfolding of both xPRAI* and fragment p_1 by addition of 6 M GuCl led to a dramatic red-shift of their fluorescence spectra, reflecting complete exposure of their Trp residues to the solvent.

(*c*) *Fragment p_1 Unfolds Reversibly and Cooperatively in Guanidinium Chloride.* Small proteins frequently unfold in a single cooperative transition (Jaenicke, 1991; Matthews, 1991). We wished to use unfolding transitions induced by GuCl as an additional criterion for autonomous folding of the isolated fragments p_1 and p_2. After denaturation by 6 M GuCl, dialysis of xPRAI* (0.2 mg/mL), of fragment p_1 (0.07 mg/mL), and of fragment p_2 (0.2 mg/mL) against buffer B yielded more than 95% recovery of folded proteins, as judged by determining the protein concentration.

Figure 7 shows that the normalized reversible unfolding transition of fragment p_1, which was measured by its ellipticity at 222 nm, was cooperative and indistinguishable from that of the intact reference protein xPRAI*. In contrast, the equilibrium unfolding and refolding of fragment p_2 followed the gradual and monotonic transition that is typical of non-cooperative processes. These data indicate that both xPRAI* and fragment p_1, but not fragment p_2, have compactly folded structures.

Assembly of the Fragment Complex. (*a*) *Properties of the Complex.* As shown in Figure 4, the apparent M_r value of the complex (≃22 000) is close to the sum of the calculated M_r values (24 885) of fragments p_1 and p_2. No assembly-incompetent fragments were detectable after stoichiometric complex formation.

The steady-state kinetic constants of the PRAI reaction catalyzed by the fragments and the fragment complex were

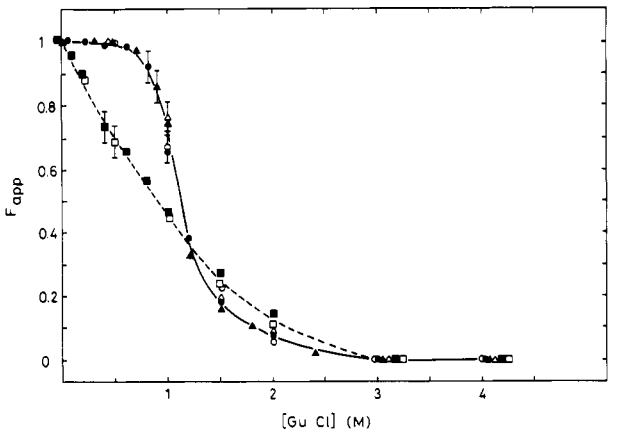

FIGURE 7: Reversible unfolding of xPRAI* and its fragments by guanidinium chloride. Ellipticities at 222 nm were measured in buffer B at 15 °C in a cell with a 0.2-cm light path as described under Experimental Procedures. F_{app} is the apparent fraction of native protein and was calculated according to the equation $F_{app} = (\theta - \theta_D)/(\theta_N - \theta_D)$ where θ represents the ellipticity at a given concentration of GuCl and θ_N and θ_D are the ellipticities in the absence and presence of 4 M GuCl, respectively (cf. Figure 5A). (▲) Unfolding of xPRAI*; (△) refolding of xPRAI*; (●) unfolding of fragment p_1; (○) refolding of fragment p_1; (■) unfolding of fragment p_2; (□) refolding of fragment p_2. Bars represent the experimental error.

Table I: Steady-State Constants of xPRAI*, Its Fragments, and the Fragment Complex[a]

protein	k_{cat} (s^{-1})	K_M^{PRA} (μM)	k_{cat}/K_M^{PRA} (μM^{-1} s^{-1})
xPRAI*	46 ± 8	3.6 ± 0.6	13
fragment p_1	$<10^{-4}$	nd[b]	
fragment p_2	$<10^{-4}$	nd	
fragment complex	25 ± 9	4.8 ± 0.6	5

[a] k_{cat} and K_M^{PRA} values measured by monitoring the disappearance of substrate (Hommel et al., 1989) (50 mM Tris-HCl buffer, pH 8.0, at 15 °C). [b] nd, not determined.

measured to monitor indirectly any differences in the spatial distribution of catalytic side chains by comparison to the reference protein xPRAI*. As seen from the values collated in Table I, k_{cat} of the fragment complex was 2-fold smaller, and K_M^{PRA} about 30% larger than the values of xPRAI*. Thus, the catalytic efficiency constant, k_{cat}/K_M^{PRA}, of the complex is only 2.5-fold smaller than that of xPRAI*. As expected, both fragments p_1 and p_2 had no PRAI activity.

(*b*) *Assembly at Equilibrium.* Since the artificial fragment complex was enzymically active, we used steady-state kinetics to determine its thermodynamic dissociation constant. Figure 8 shows that addition of increasing concentrations of fragment p_2 to a constant concentration of fragment p_1 led to maximal activity at the stoichiometric ratio. The equilibrium titration data fitted well to a value of $K_d = 0.2$ μM. Alternatively, a p_1/p_2 fragment complex was also obtained by dialyzing a stoichiometric mixture of the fragments unfolded at 4 M GuCl. It had the same kinetic constants as the complex assembled from prefolded fragments.

(*c*) *Fragment Association Kinetics.* The kinetics of the association of the fragments were measured to gain insight into the mechanism of assembly. The recovery of enzymic activity after mixing the two fragments at a stoichiometric ratio was a slow exponential process (Figure 9) without an initial burst of activity. k_{obs} [$(4.0 \pm 0.5) \times 10^{-3}$ s^{-1}] is in the same time range as the slow refolding rate constant of xPRAI* ($k_{obs} = 3.5 \times 10^{-3}$ s^{-1}; Luger et al., 1989). Figure 10 shows that the first-order reactivation process does not depend on the concentration of fragment p_2 up to a 20-fold excess.

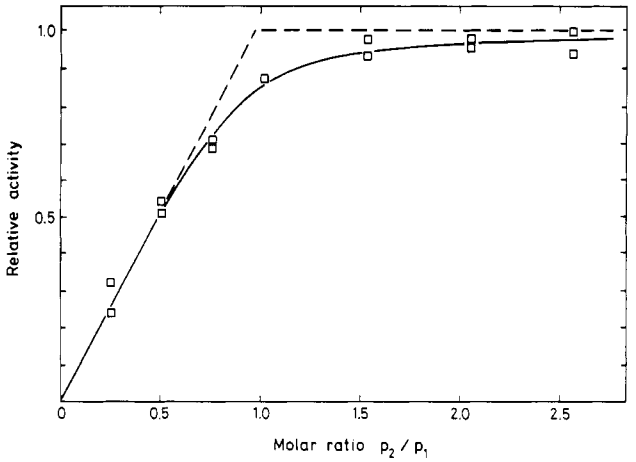

FIGURE 8: Determination of the equilibrium constant of fragment assembly. Titration of fragment p_1 (5.8 μM) with aliquots of a solution of fragment p_2 (0.285 mM) in buffer B at 15 °C. (□) PRAI activity measured as described under Experimental Procedures and normalized by the final plateau value. Results from two independent experiments. (—) Fitted curve with $K_d = 0.2$ μM. (---) Theoretical curve for infinite binding affinity.

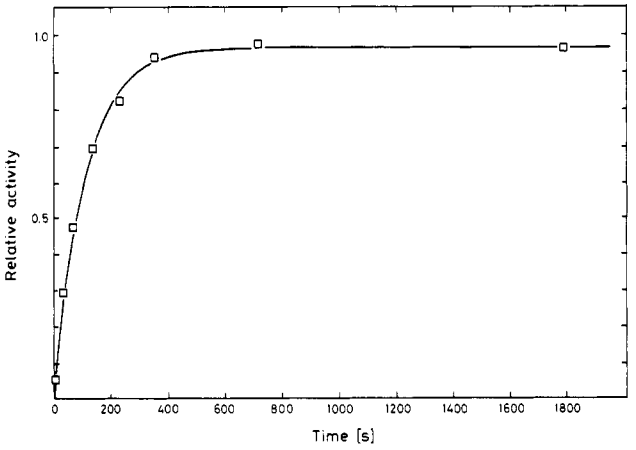

FIGURE 9: Fragment assembly is a single-exponential process. Progress curve of the recovery of enzyme activity. The association was initiated by mixing fragment p_1 manually with fragment p_2 in buffer B at 15 °C, and followed by measuring PRAI activity. (□) Both fragments at an identical final concentration of 7 μM. (—) Fitted exponential curve with $k_{obs} = 4.0 \times 10^{-3}$ s^{-1}.

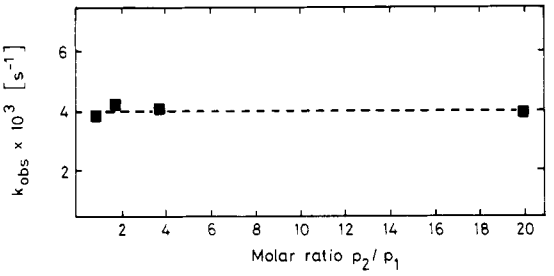

FIGURE 10: k_{obs} is independent of the concentration of fragment p_2. (■) Fragment p_1 (7 μM in buffer B) was mixed to different molar ratios with the p_2 fragment.

(*d*) *Assembly of Fragment p_1 and the DHFR/Xa/p_2 Fusion Protein.* The fusion protein possesses the same turnover number in the DHFR reaction as mDHFR (data not shown), proving that its carboxyl-terminal extension by the p_2 region does not prevent correct folding of the mDHFR domain. It is of interest whether covalent attachment of fragment p_2 to a folded protein induces structure in the attached fragment. To test this possibility, we prepared a stoichiometric mixture of fragment p_1 and the fusion protein. It was catalytically

active in the PRAI reaction, but the relatively large dissociation constant (K_d = 1.5 μM, compared to K_d = 0.2 μM of the fragment complex) led to partial dissociation of the complex under conditions of the enzyme assay and during gel filtration. The rate-limiting constant of the assembly reaction (k_{obs} = 8.0×10^{-4} s^{-1}) was about 20-fold smaller than that observed for the free fragments. In summary, fusion of fragment p_2 via a flexible linker to compactly folded mDHFR decreased its assembly competence.

Discussion

The expression of designed fragments of the TRP1 gene led to the large amino-terminal fragment p_1 in a straightforward manner, but production of the small carboxyl-terminal fragment p_2 needed the familiar detour via a fusion protein (Nagai et al., 1985; Nagai & Thogersen, 1987). The soluble, monomeric fragments assembled spontaneously to a compact stoichiometric complex that was almost as active enzymically as the intact reference protein xPRAI*. However, only fragment p_1 had the properties of a compact protein with a defined fold, and therefore seems to be crucial for understanding the folding of xPRAI*.

The conserved and catalytically important residues of PRAI (cf. Figure 1) are located in the $\beta\alpha$ units 1, 2, 6, 7, and 8 (Priestle et al., 1987; Wilmanns et al., 1992; Eder, Urfer, Zapun, and Kirschner, unpublished work). If the interactions between these crucial secondary structural elements were perturbed by cleaving the loop of xPRAI* between α_6 and β_7, the values of k_{cat} and K_M^{PRA} would be expected to deteriorate. Therefore, the minor differences between the kinetic constants of xPRAI* and the fragment complex indicate that their central 8-fold $\beta\alpha$ cores as well as the loops between β strands and α helices at the respective active sites are almost identical.

Why is θ_{222} of the complex smaller than that of xPRAI* (cf. Figure 5A)? Although quantitative estimates of the fraction of residues in either the α-helix or the β-strand conformation (Yang et al., 1986) were not feasible, the complex appears to have less α-helical structure than xPRAI*. Cleavage of the loop of xPRAI* between α_6 and β_7 (or assembling the active complex from fragments p_1 and p_2) may destabilize α_6 somewhat but not to the extent of perturbing the catalytically important loop between β_6 and α_6. The decreased amplitude of the near-UV CD spectrum of the complex (Figure 5B) and the shift of its tryptophan fluorescence spectrum to longer wavelengths (Figure 6) might be due to an altered environment of W163, which is only seven residues away from the carboxyl terminus of fragment p_1.

The fragment p_2 appears to be only partially folded. It is less compact than expected for a native protein of the same size (Figure 4), possesses no detectable near-UV CD spectrum, and unfolds only gradually as the concentration of GuCl is increased (Figure 7). Its far-UV CD spectrum is consistent with an almost total loss of α helix but retention of some β structure (Yang et al., 1986).

The fragment p_1 shares several important properties with xPRAI*. It is compact, and unfolds cooperatively. Both its far-UV CD and tryptophan fluorescence spectra are different from those of xPRAI* but are similar to those of the fragment complex. Perhaps the fragment p_1 has the overall structure of a 6/8 $\beta\alpha$ barrel, in which the exposed hydrophobic area that is complementary to the inner surface of the missing p_2 region is shielded from solvent somehow. Cellobiohydrolase II is a precedent for a shielded 6/8 $\beta\alpha$ barrel (Rouvinen et al., 1990). The region $\alpha_7\beta_8\alpha_8$ is missing, and β_7 makes a skewed connection between β_1 and β_6. In the case of fragment p_1 of yPRAI, the amphipathic helix α_6 and its carboxyl-terminal extension could provide the necessary shielding.

It is possible that such a structure would retain most of the central β-strand residues but would lose the α-helical conformation of some external residues. For example, partial unfolding of α_1, which has lost its neighbor α_8, could explain the difference between the far-UV spectra of xPRAI* and fragment p_1. A new chiral environment for either W_{157} (on α_6) or W_{163} (on the carboxyl-terminal extension of α_6), or both, could also explain in general terms the inverse amplitude of the near-UV CD spectrum of fragment p_1. However, without direct data on its structure, one cannot exclude alternative models for fragment p_1.

Enzymic activity is acquired with a slow progress curve consisting of a single exponential after mixing the fragments p_1 and p_2 (Figure 9). When the refolding of xPRAI, which is closely related to xPRAI* (Luger et al., 1990), was followed by recovery of enzymic activity, a single-exponential progress curve was also observed with a rate constant that is remarkably similar to that of fragment assembly. This slow process was also monitored by tryptophan fluorescence, but it was preceded by a rapid ($t_{1/2}$ < 1 s) rise of fluorescence. This observation suggests that a collapsed intermediate (Kuwajima, 1989; Kim & Baldwin, 1990) accumulates prior to the rate-determining step of recovering enzymic activity. Similar experiments with fragment p_1 (data not shown) revealed that its slow refolding phase occurred 5-fold more rapidly than that of xPRAI. On the basis of these facts and the similar overall stabilities of fragment p_1 as well as the intact protein toward GuCl, we propose that parts of the native $(\beta\alpha)_8$ barrel are formed first on the folding pathway of xPRAI, before the complete barrel structure is attained. The slow refolding phase of xPRAI would therefore reflect either the rearrangement of nonnative substructures required for shielding the exposed hydrophobic cavity of the p_1 region or the folding of the p_2 region, or both.

The fragment p_2 does not have sufficient intrinsic stabilizing interactions to fold completely by itself. On one hand, if a folding unit is defined as an independently folding region (i.e., all determinants that direct its folding reside within its own amino acid sequence; Baldwin, 1989), then the region β_7–α_8 of xPRAI* is probably not a folding unit. On the other hand, if the folding unit is defined in the context of the intact polypeptide chain during the lifetime of the compact intermediate state, the capacity of the small fragment p_2 to fold predominantly to the native format all by itself may not be directly relevant. If an equilibrium exists in xPRAI between natively structured and dissordered forms of the region p_2, then the folded region p_1 could act as a template for structure formation of the region p_2.

This working hypothesis on the mechanism of fragment complementation of xPRAI* is similar to the mechanism of refolding of the α subunit of tryptophan synthase. At equilibrium, the less stable carboxyl-terminal region (α_6–α_8) unfolds at low urea concentrations, followed by the unfolding of the more stable amino-terminal region (β_1–β_6) at high urea concentrations (Miles et al., 1982; Beasty & Matthews, 1985). A 6 + 2 refolding mechanism with two different folding units was inferred from kinetic studies, and it was shown that the rate-limiting step of refolding, which occurs late on the folding pathway, is the merging of the two folding units (Beasty et al., 1986; Tweedy et al., 1990; Chrunyk & Matthews, 1990).

In indoleglycerolphosphate synthase, ePRAI, and the α subunit of tryptophan synthase, the region p_2 contains a greater percentage of identical residues than the region p_1 (Wilmanns et al., 1991). It is interesting that the region p_2 in two out of three of the above enzymes seems to have maintained the

same functional role (i.e., the binding of the phosphate moiety of the substrate) as well as its role in protein folding. These findings further support the hypothesis that PRAI and the α subunit of tryptophan synthase have diverged from a common ancestor.

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