Phosphoribosyl anthranilate isomerase from *Thermotoga maritima* is an extremely stable and active homodimer*

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Abstract

The metabolism of hyperthermophilic microorganisms can function properly at temperatures close to 100 °C. It follows that they are equipped with both thermostable enzymes and mechanisms that handle labile metabolites. We wanted to understand how stable and active phosphoribosyl anthranilate isomerase (tPRAI) from the hyperthermophile *Thermotoga maritima* is at its optimum growth temperature of 80 °C, and how its thermolabile substrate, *N*-(5'-phosphoribosyl)-anthranilate (PRA), is protected from rapid decomposition. To this end, the *trpF* gene of *T. maritima* was expressed heterologously in *Escherichia coli* and tPRAI was purified. In contrast to most PRAIs from mesophiles, which are monomers with the eightfold $\beta\alpha$ (or TIM) barrel fold, tPRAI is a homodimer. It is strongly resistant toward inactivation by temperatures up to 95 °C, by acidification to pH 3.2, and by proteases in the presence and absence of detergents. tPRAI is about 35-fold more active at its physiologic temperature than is the enzyme from *E. coli* (ePRAI) at 37 °C. This high catalytic efficiency of tPRAI is likely to compete successfully with the rapid spontaneous hydrolysis of PRA at 80 °C. Thus, with respect to both stability and function, tPRAI appears well adapted to the extreme habitat of *T. maritima*. Single crystals of tPRAI have been obtained that are suitable for X-ray analysis at high resolution.

Keywords: ($\beta\alpha$)₈-barrel proteins; hyperthermophile; labile substrate; steady-state kinetics; thermostability; tryptophan biosynthesis

Enzymes from thermophilic organisms are well suited for investigating structure, stability, and evolution of proteins. The hyperthermophile *Thermotoga maritima* (optimum growth temperature: 80 °C) occupies one of the earliest and most slowly evolving

branches within the bacterial domain of life (Achenbach-Richter et al., 1987). Although it was discovered only 10 years ago (Huber et al., 1986), about a dozen thermostable enzymes either have been isolated directly from *T. maritima* cells (Wrba et al., 1990a, 1990b; Engel et al., 1992; Schurig et al., 1995a, 1995b) or expressed heterologously and purified from *Escherichia coli* cells that had been transformed with the respective genes (Tiboni et al., 1989; Liebl et al., 1992; Sanangelantoni et al., 1992; Ostendorp et al., 1993; Tomschy et al., 1993; Darimont & Sterner, 1994; Vieille et al., 1995; Winterhalter et al., 1995).

Tryptophan biosynthesis from chorismic acid comprises seven catalytic functions (Crawford, 1989). The fourth step in this key metabolic pathway, the conversion of *N*-(5'-phosphoribosyl)-anthranilate to 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate, is catalyzed by phosphoribosyl anthranilate isomerase:

This enzyme belongs to the family of TIM- or ($\beta\alpha$)₈-barrel proteins (Priestle et al., 1987; Wilmanns et al., 1992), which represent the most frequently encountered topology among single-

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Abbreviations: tPRAI, phosphoribosyl anthranilate isomerase from *T. maritima*; PRA, *N*-(5'-phosphoribosyl)-anthranilate; *trpF*, gene encoding phosphoribosyl anthranilate isomerase; TIM, triosephosphate isomerase; ePRAI, monofunctional PRAI domain of the bifunctional enzyme indoleglycerol phosphate synthase:phosphoribosyl anthranilate isomerase from *E. coli*; CdRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate; IPTG, isopropyl- β -D-thiogalactopyranoside; LB medium, Luria-Bertani medium; OD₆₀₀, optical density at 600 nm; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; MES, 2-morpholino-ethanesulfonic acid; yPRT, anthranilate phosphoribosyl transferase from yeast; tPRT, C-terminal PRT domain of the fusion protein glutamine amidotransferase:anthranilate phosphoribosyl transferase from *T. maritima*; PRPP, 5'-phosphoribosyl-1-diphosphate; eIGPS, monofunctional IGPS domain of the bifunctional enzyme indoleglycerol phosphate synthase:phosphoribosyl anthranilate isomerase from *E. coli*; rCdRP, reduced CdRP.

domain protein structures (Reardon & Farber, 1995). Recently, the entire tryptophan operon of *T. maritima* has been cloned and sequenced (Kim et al., 1993; Sterner et al., 1995). It has the gene order *trpE*(G·D)CFBA, with *trpF* coding for tPRAI. Here we report the heterologous expression, purification, characterization, and crystallization of tPRAI. In contrast to the known monomeric PRAIs from mesophilic organisms (Enatsu & Crawford, 1968; Hoch et al., 1969; Creighton, 1970; Braus et al., 1988; Eberhard et al., 1995), tPRAI is a homodimer that is extremely stable toward heat, acidic pH, and proteolytic attack. Due to its high activity at 80 °C, tPRAI can outstrip the rapid hydrolysis of its thermolabile substrate PRA under physiologic conditions.

Results

Production and purification of tPRAI

The *trpF* gene encoding tPRAI (Sterner et al., 1995) was subcloned into the expression vector pQE-60. Proteins are expressed in *E. coli* from this plasmid under the control of a *lac* promoter/repressor system, in which expression can be induced by IPTG (Bujard et al., 1987; Stüber et al., 1990). About equal amounts of tPRAI were found in the insoluble and soluble fractions of the cell-free extract of induced transformants (Fig. 1). Only the latter fraction was used for purification, which was followed by both enzyme assays (Kirschner et al., 1987) and SDS-PAGE (Table 1; Fig. 1). In the first step, we took advantage of the thermostability of the protein to remove most of the host proteins by heating the crude extract for 10 min at 80 °C. Further purification steps included ion exchange chromatography on DEAE-Sepharose and gel filtration chromatography on Sephacryl S-200. The final solution was concentrated and yielded needle-shaped crystals of >99%

Fig. 1. Purification of tPRAI from transformed *E. coli* cells monitored by electrophoresis on 12.5% polyacrylamide gels in the presence of SDS. Proteins were stained with Coomassie blue. Lane A, soluble fraction of the cell extract; lane B, insoluble fraction of the cell extract (pellet); lane C, supernatant of heat step; lane D, eluate from DEAE-Sepharose column; lane E, eluate from Sephacryl S-200 column; lane F, dissolved crystals; lanes M, standard proteins, with molecular weights given in kDa on the right.

Table 1. Purification of tPRAI from 96 g (wet weight) transformed *E. coli* cells

	Volume (mL)	Protein (mg)	Spec. activity (IU/mg)	Yield (%)
Crude extract	392	2,557	ND ^a	ND ^a
80 °C supernatant	373	364	3.80	100
DEAE-Sepharose eluate	124	73	12.80	69
Sephacryl S-200 eluate	35	45	13.80	46
Crystals	4	22	14.30	23

^aND, not determined.

purity spontaneously overnight at 0 °C. These crystals were washed and dissolved in buffer, giving the stock solution used for biochemical studies and production of crystals suitable for protein crystallographic determination of the tPRAI structure.

Sedimentation equilibrium runs gave a molecular weight of 49.6 kDa at pH 7.0 (Fig. 2). Because the calculated molecular weight for the monomer is 23.04 kDa (Sterner et al., 1995), tPRAI is a homodimer. The molar extinction coefficient for the dimer is $\epsilon_{280} = 29,400 \text{ M}^{-1} \text{ cm}^{-1}$, ($^{0.1\%}A_{280} = 0.64 \text{ cm}^2 \text{ mg}^{-1}$), as determined by second derivative spectroscopy (Levine & Federici, 1982).

Steady-state kinetics

Entire progress curves of the reaction of enzymatically produced PRA (Hommel et al., 1995) catalyzed by tPRAI and ePRAI were recorded at various temperatures. Table 2 presents the steady-state catalytic constants obtained for tPRAI at 25 °C, 45 °C, and 60 °C, in comparison to those obtained for ePRAI at 25 °C and 37 °C. Remarkably, the value of the catalytic efficiency parameter

Fig. 2. tPRAI is a homodimer between pH 7.0 and pH 3.2. Sedimentation equilibrium runs at 20 °C and at 18,000 rpm were performed with 26 μM of tPRAI in 50 mM potassium phosphate containing 0.2 mM EDTA and 0.2 mM DTT. pH 7.0 (○); pH 4.5 (△); pH 3.2 (□). Inset: Plot of $\ln(\text{absorbance})$ versus r^2 . The calculated molecular weights are 49.6 kDa at pH 7.0, 48.6 kDa at pH 4.5, and 49.0 kDa at pH 3.2.

Table 2. Temperature dependence of steady-state kinetic constants of tPRAI and ePRAI^a

	T (°C)	k_{cat} (s ⁻¹)	K_M^{PRA} (μM)	k_{cat}/K_M^{PRA} (μM ⁻¹ s ⁻¹)	K_P^{CdRP} (μM) ^b
tPRAI	25	3.7	0.28	13.3	0.21
	45	13.5	0.39	34.4	0.46
	60	38.5	0.73	52.1	ND ^c
	80	116.8 ^d	1.03 ^d	113.4	ND
ePRAI	25	34.5	12.2	2.8	ND
	37	74.7	22.9	3.3	ND

^aBuffer conditions: 50 mM Tris/HCl, 4 mM K₂MgEDTA, 2 mM DTT, pH 7.5, at the respective temperature.

^bProduct inhibition constant.

^cND, not determined.

^dExtrapolated from Arrhenius plots.

k_{cat}/K_M^{PRA} of tPRAI (13.3 μM⁻¹ s⁻¹) is approximately fivefold larger at 25 °C than that of ePRAI (2.8 μM⁻¹ s⁻¹). Arrhenius plots for both k_{cat} and K_M^{PRA} of tPRAI allow an extrapolation of these catalytic constants to higher temperatures. Accordingly, the values for k_{cat} and K_M^{PRA} at 80 °C, the optimum growth temperature of *T. maritima* (Huber et al., 1986), are 116.8 s⁻¹ and 1.03 μM; consequently, the resulting catalytic efficiency parameter k_{cat}/K_M^{PRA} equals 113.4 μM⁻¹ s⁻¹ (Table 2).

Is PRA channeled in a stable complex between tPRAI and tPRT?

In order to compare the stability of the substrate at the growth temperatures of *E. coli* and *T. maritima*, the hydrolysis of PRA to anthranilic acid and ribose-5-phosphate was measured at various temperatures in potassium phosphate buffer at pH 7.5. The decomposition of PRA is an exponential process with the following half lives (first-order rate constants): 449 s (1.54·10⁻³ s⁻¹) at 25 °C, 291 s (2.38·10⁻³ s⁻¹) at 37 °C, 144 s (4.82·10⁻³ s⁻¹) at 50 °C, 84 s (8.27·10⁻³ s⁻¹) at 60 °C, and 39 s (1.74·10⁻² s⁻¹) at 80 °C. Creighton (1968) reported the half life of PRA to be 210 s at 37 °C and pH 7.67. Thus, PRA is degraded rapidly at elevated temperatures. We therefore tested whether it might be channeled by complex formation between anthranilate phosphoribosyl transferase from *T. maritima* (tPRT) and tPRAI. tPRT is the dimeric C-terminal domain of the fusion protein glutamine amidotransferase:tPRT (a gift from A. Ivens; cf. Kim et al., 1993; Sterner et al., 1995). To this end, the molecular weights of the dimeric enzymes were analyzed by sedimentation equilibrium runs at a concentration of 5 μM, both separately and after mixing. Isolated tPRAI and tPRT showed molecular weights of 49 kDa and 76 kDa, respectively. After mixing the enzymes, two separate mass distributions were found, corresponding to the molecular weights of the isolated proteins (data not shown).

Stability measurements

Attempts to unfold tPRAI reversibly with guanidinium chloride at neutral or acidic pH values were not successful. The protein unfolded very slowly, with a strong tendency to aggregate in the transition region at about 2.5 M guanidinium chloride (data not

shown). Therefore, the stability of tPRAI relative to that of ePRAI was measured operationally by measuring the rate of its irreversible inactivation by heat, as well as its resistance toward acidification and proteases.

The irreversible loss of tPRAI activity when incubated at elevated temperatures is an exponential process (Fig. 3) of unknown mechanism. The half lives of tPRAI (in 50 mM potassium phosphate at pH 7.0) at 85 °C, 91.5 °C, and 95 °C are 310 min, 50 min, and 21 min, respectively. The activation energy of unfolding for tPRAI was calculated to be 296 kJ/mol from an Arrhenius plot. In comparison, ePRAI is much less thermostable: at 67 °C, it has a half-life of 120 min (data not shown).

The stability of tPRAI was also probed by acidification (Fink et al., 1994). To this end, the molecular weight of tPRAI was determined by sedimentation equilibrium runs in the analytical ultracentrifuge at pH 7.0, pH 4.5, and pH 3.2. The corresponding molecular weights were 49.6, 48.6 and 49.0 kDa, respectively, showing that dimeric tPRAI is remarkably resistant to acid-induced dissociation (Fig. 2).

The binding of the competitive inhibitor rCdRP is a very sensitive test for the integrity of the active site of tPRAI (Bisswanger et al., 1979; Cohn et al., 1979; Eberhard et al., 1995; Hommel et al., 1995). Binding isotherms were determined at both pH 3.2 and pH 7.0 (Fig. 4). tPRAI binds rCdRP with the expected stoichiometry (2 mol rCdRP per mol dimeric tPRAI) in a non-cooperative manner, and with high affinity (K_d^{rCdRP} = 4.7 μM at pH 3.2, 0.50 μM at pH 7.0). In accordance with these results, the far-UV CD spectrum of tPRAI is practically identical at pH 3.2 and pH 7.0 (Fig. 5A). In contrast, the far-UV CD spectrum of ePRAI is shifted toward lower wavelengths at pH 3.2 compared with that at pH 7.0. The near-UV CD spectrum of tPRAI, between 255 and 270 nm, does not depend on pH, but between 270 and

Fig. 3. tPRAI is extremely thermostable. tPRAI was incubated at the indicated temperatures in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM DTT. Samples were taken after the indicated time intervals and chilled on ice. The residual activities were measured subsequently at 25 °C by recording and fitting entire progress curves as described in Materials and methods. Fits of the data points to a single exponential decay gave the following half lives: 310 min at 85 °C, 50 min at 91.5 °C, and 21 min at 95 °C.

Fig. 4. tPRAI is functionally intact under acidic conditions. The reversible binding of the competitive inhibitor rCdRP to dimeric tPRAI ($1.07 \mu\text{M}$) was measured by fluorimetric titration at 20°C and at pH 7.0 (○) and at pH 3.2 (□). Buffer: 50 mM potassium phosphate containing 0.2 mM EDTA and 0.2 mM DTT. Fitting of the data points according to Eberhard et al. (1995) and under the assumption of two binding sites of rCdRP per dimer of tPRAI gave K_d values of $0.50 \mu\text{M}$ at pH 7.0 and $4.7 \mu\text{M}$ at pH 3.2.

290 nm, it is reduced by acidification (Fig. 5B). The near-UV CD spectrum of ePRAI is completely lost at acidic pH. These results show that tPRAI retains much of its native structure down to a pH value of 3.2, whereas the tertiary structure of ePRAI is largely disordered at that pH value.

Fig. 5. The structure of tPRAI is more resistant to acidification than the structure of ePRAI. CD spectra of tPRAI and ePRAI were recorded at 22°C in 50 mM potassium phosphate containing 1 mM EDTA and 1 mM DTT at pH 7.0 or pH 3.2. tPRAI at pH 7.0 (○) and at pH 3.2 (□); ePRAI at pH 7.0 (●) and at pH 3.2 (■). **A:** Far-UV CD spectra: $d = 0.1 \text{ cm}$; concentrations of both tPRAI and ePRAI were $9 \mu\text{M}$. **B:** Near-UV CD spectra: $d = 5 \text{ cm}$; concentrations of tPRAI and ePRAI were $16 \mu\text{M}$ and $10 \mu\text{M}$, respectively.

In order to test the stability of tPRAI toward proteolytic digestion, the enzyme was treated with several proteases (trypsin, GluC, LysC, and ArgC), as well as under a number of different conditions. As judged by SDS-PAGE, proteolytic cleavage of tPRAI was not observed with any of these proteases in 100 mM Tris/acetate buffer at pH 7.8, neither after 60 min incubation at 50°C , nor in the presence of 0.5% SDS under the same conditions. In accordance with these results, the activity of tPRAI before and after exposure to various proteases was unchanged. Incubation of tPRAI with pepsin for 90 min in 50 mM potassium phosphate at pH 3.2 did not result in any measurable proteolytic degradation of tPRAI, whereas ePRAI was completely digested under identical conditions (data not shown).

Crystals suitable for X-ray diffraction

tPRAI was crystallized for X-ray diffraction studies. The hexagonal rods (Fig. 6) grew to a maximum size of $0.25 \times 0.25 \times 1.5 \text{ mm}^3$. The crystals were suitable for X-ray analysis and diffracted to better than $1.8 \text{ \AA}$ resolution. They belong to the space group $P6_22$ or its enantiomer $P6_522$, with cell dimensions $a = b = 68.4 \text{ \AA}$, $c = 185.4 \text{ \AA}$ and $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. Assuming that there is one monomer in the asymmetric unit, the solvent content of the crystals amounts to 45%. The data are 98.8% complete in the resolution range between $10.0 \text{ \AA}$ and $2.0 \text{ \AA}$. The overall R -factor, based on intensities of the symmetry-equivalent reflections, is 6.4%.

Discussion

Stability and association state of tPRAI

T. maritima, which forms one of the deepest branches within the phylogenetic domain of the Bacteria (Woese et al., 1990), has an upper growth limit of 90°C (Huber et al., 1986). It therefore

Fig. 6. Crystals of tPRAI grown after two weeks at 19.4 mg/mL in 0.1 M MES buffer, 0.05 M potassium phosphate, pH 6.5, containing 1.5 M ammonium sulphate and 10 mM CoCl_2 at 22 °C. The size of the crystals is about $0.2 \times 0.2 \times 1.5 \text{ mm}^3$.

qualifies as a hyperthermophile (Stetter et al., 1990; Adams, 1993). Because metabolic reactions must be catalyzed also under these extreme conditions, the enzymes of this organism have to be equally thermostable. tPRAI, which has a half life of 50 min at 91.5 °C (Fig. 3), has enough *intrinsic* stability to function at physiologic temperatures. Enzymes from *T. maritima* involved in glycolysis (Jaenicke, 1996) and sugar metabolism (Vieille et al., 1995; Winterhalter et al., 1995) show comparatively high thermostabilities. Several hyperthermophilic Archaea appear to need *extrinsic* factors, such as 2,3-diphosphoglycerate (Hensel & König, 1988), dimyo-inositolphosphate (Scholz et al., 1992; Martins & Santos, 1995), and mannosylglycerate (Martins & Santos, 1995), to stabilize enzymes for proper functioning at physiologic temperatures (Hensel et al., 1994). It is not clear whether *T. maritima* contains similar factors and whether they would further stabilize tPRAI.

The high thermostability of tPRAI goes along with very slow unfolding in guanidinium chloride and pronounced resistance toward both proteolytic attack and low pH values (Fig. 5). Similarly, rubredoxin from the archaeon *Pyrococcus furiosus* is highly thermostable and extremely resistant to acid-induced denaturation (Cavagnero et al., 1995). It has also been shown in a number of cases that thermophilic proteins are more resistant to proteolytic attack than their mesophilic homologues (Fontana, 1988). These observations support the view that proteins from hyperthermophiles are not only protected against heat, but against denaturing conditions in general.

Whereas the PRAIs from yeast (Braus et al., 1988), *Pseudomonas putida* (Enatsu & Crawford, 1968), and *Bacillus subtilis* (Hoch et al., 1969) are monomeric and monofunctional, PRAI from *E. coli* is part of a bifunctional polypeptide chain (eIGPS: PRAI), which also catalyzes the subsequent step (IGPS) in tryptophan biosynthesis (Creighton, 1970). However, the separately expressed monofunctional domains (ePRAI and eIGPS) are monomers and show practically identical catalytic properties as in their native bifunctional context (Eberhard et al., 1995). Therefore, the homodimeric structure of tPRAI is unique among all known PRAIs. Higher association states have been found for several extremely thermostable glycolytic enzymes in comparison to their counterparts from mesophilic microorganisms: Phosphoglycerate kinase, which is a monomer in all investigated mesophiles, is a dimer in both the hyperthermophilic Archaea *P. furiosus* and *Meth-*

anothermus fervidus (Hess et al., 1995); in *T. maritima*, an octameric fusion protein phosphoglycerate kinase-triosephosphate isomerase is found in addition to a monomeric version of phosphoglycerate kinase (Schurig et al., 1995a); triosephosphate isomerase, which is dimeric in all investigated mesophiles, is a tetramer in *P. furiosus* (Kohlhoff et al., 1996). It appears that oligomerization is crucial for thermostability in these cases. However, monomeric phosphoglycerate kinase from *T. maritima* is as thermostable ($T_m \sim 100^\circ\text{C}$) as the octameric phosphoglycerate kinase-triosephosphate isomerase fusion protein (Schurig et al., 1995a).

Catalytic properties of tPRAI

Several enzymes from *T. maritima* and other hyperthermophiles are almost inactive at room temperature (Jaenicke, 1996). In contrast, tPRAI is highly active at 25 °C, especially when compared with the corresponding enzyme from *E. coli* (Table 2). This result is in accordance with in vivo complementation experiments, which showed that, at 37 °C and on selective medium, *trpF* from *T. maritima* rapidly confers growth ability to *E. coli* strains lacking *trpF* (Sterner et al., 1995). In general, low activity of thermostable enzymes at room temperature is explained by their conformational rigidity, which is relieved at the higher physiologic temperatures. A well-studied example is glyceraldehyde 3-phosphate dehydrogenase from *T. maritima*, which shows a slow H-D exchange rate of its peptide amide protons at 25 °C (Wrba et al., 1990b). Moreover, its activity at room temperature can be increased by adding low concentrations of guanidinium chloride (Rehaber & Jaenicke, 1992). Presumably, the activation is due to flexibility of parts of the active site.

The inability of various proteases to cleave tPRAI suggests that its high activity at room temperature is not due to a high overall conformational flexibility of the enzyme (Fontana, 1988). However, the mechanism of the catalyzed reaction might provide a possible explanation for the high efficiency of tPRAI at room temperature. PRAI catalyzes an Amadori rearrangement, which requires general acid-base catalysis (Yaylayan & Huyghues-Despointes, 1994), and probably no major conformational rearrangements at the active site.

Several metabolites decay with very short half lives at elevated temperatures (Larralde et al., 1995). This problem applies particularly to PRA, the substrate of tPRAI. One strategy to overcome this problem would be to hinder PRA from entering the bulk solution by forming of a complex between the metabolic source (tPRT) of PRA and its sink (tPRAI). However, as in the case of yeast PRAI and PRT (Hommel et al., 1989), we were unable to detect a stable complex between these two enzymes from *T. maritima* by analytical ultracentrifugation, at least at concentrations of 5 μM for each dimeric protein. Similar results were obtained with the pair of enzymes that generate and transform phosphoribosylamine, which is the labile metabolite in the first step of de novo purine biosynthesis, having a half life of 5 s at 37 °C and pH 7.5 (Rudolph & Stubbe, 1995). Because a stable complex between phosphoribosylpyrophosphate amidotransferase and glycynamide ribonucleotide synthetase was not detectable, the authors postulated that the labile metabolite is transferred during the lifetime of a transient complex (Srivastava et al., 1989) between the two enzymes. However, in the case of tPRAI, the rapid hydrolysis of the substrate might be outstripped by other mechanisms (see below).

Several enzymes from hyperthermophiles show comparable activities to their counterparts from mesophiles at the respective physiologic temperatures (Jaenicke, 1996). Importantly, as esti-

mated from a comparison of k_{cat}/K_M^{PRA} values, tPRAI is more active by a factor of about 35 at the optimum growth temperature (80 °C) of *T. maritima* than is ePRAI at the optimum growth temperature (37 °C) of *E. coli* (Table 2). The physiologic significance of this unusually high relative activity of tPRAI may be to compete efficiently with the unproductive spontaneous hydrolysis of the extremely labile substrate PRA at elevated temperatures (Creighton, 1968; this work).

At the likely low PRA concentrations under physiologic conditions ($[PRA] < K_M^{PRA}$), the velocity of conversion (v_c) of PRA to CdRP by PRAI is given by the pseudo first-order equation:

$$v_c = -d[PRA]/dt = (k_{cat}/K_M^{PRA}) \cdot [E_0] \cdot [PRA],$$

where $[E_0]$ is the total concentration of PRAI active sites. The velocity of the competing spontaneous hydrolysis of PRA (v_h) is given by the first-order equation:

$$v_h = -d[PRA]/dt = k_h \cdot [PRA].$$

The ratio between the velocities of both processes is:

$$v_c/v_h = ((k_{cat}/K_M^{PRA})/k_h) \cdot [E_0].$$

The concentration of PRAI active sites (i.e., $[E_0]$) in derepressed cells of *E. coli* at 37 °C equals 2.2 μM (Creighton & Yanofsky, 1966). Under these conditions, and with the measured values of k_{cat}/K_M^{PRA} (3.3 $\mu\text{M}^{-1} \text{s}^{-1}$) of ePRAI and k_h ($2.38 \cdot 10^{-3} \text{s}^{-1}$), $v_c/v_h \sim 2800$. Even when $[E_0]$ drops 20-fold in repressed cells of *E. coli* (Jackson & Yanofsky, 1973), the value of v_c/v_h (=140) is high enough to efficiently capture PRA for tryptophan biosynthesis. The values of k_{cat}/K_M^{PRA} (113.4 $\mu\text{M}^{-1} \text{s}^{-1}$) of tPRAI and k_h ($1.74 \cdot 10^{-2} \text{s}^{-1}$) at 80 °C show that *T. maritima* would require only 20% of the enzyme levels of *E. coli* at 37 °C to achieve comparable values of v_c/v_h . These calculations indicate that the productive processing of PRA does not require transient complex formation between PRAI and PRT, either in *E. coli* or in *T. maritima*.

Materials and methods

DNA manipulation and sequence analysis

Preparation of DNA probes, digestion with restriction endonucleases, agarose gel filtration, and DNA ligation were performed as described by Sambrook et al. (1989). The CircumVent Thermal Cycle Sequencing kit from New England Biolabs was used for DNA sequencing. PCRs were performed in the Trio-block from Biometra (Göttingen, Germany), using thermostable polymerases either from *Thermus aquaticus* (Boehringer, Mannheim, Germany) or from *P. furiosus* (Stratagene, Heidelberg, Germany). Oligonucleotides were purchased from Microsyn (Windisch, Switzerland). Proteases (GluC, LysC, ArgC, trypsin, pepsin) were obtained from Boehringer (Mannheim, Germany).

Expression of *T. maritima* *trpF* in *E. coli* and purification of the recombinant tPRAI

The *trpF* gene of *T. maritima* was amplified by PCR, using the plasmid pUN121/III (Stern et al., 1995) as a template. The oligonucleotide 5'-AGACCATGGTCAGAGTGAAAATC-3' with a

Nco I-site (in bold) was used as the 5'-primer, and the oligonucleotide 5'-AGTAAGCTTTCATTCTCACAACC-3' with a *Hind* III-site (in bold) as the 3'-primer. These two restriction sites permitted cloning of the fragment into the expression plasmid pQE-60 (Qiagen, Chatsworth, USA) to yield the clone pQE-60TmtrpF. The insert was subjected to DNA sequencing to exclude inadvertent PCR mutations. In the pQE expression system, genes are transcribed from the *lac* promoter after induction with IPTG, which inactivates the *lac* repressor encoded by the repressor plasmid pREP4. Expression was conducted in SG 200-50 cells (Casabadian, 1976), which are a *lonA*⁻ (Tn5) derivative of strain MC 4110 (F, $\Delta lacU$ 169, *araD*139, *rpsL*, *relA*, *thiA*, *flbB*). Transformed cells were grown overnight at 37 °C in 400 mL LB medium supplemented with 0.1 mg/mL ampicillin (for maintenance of pQE-60TmtrpF) and 0.025 mg/mL kanamycin (for maintenance of pREP4). This culture was used to inoculate a fermenter (Bioengineering, Wald, Switzerland) containing 37 L of the same medium and incubated at 30 °C until OD₆₀₀ attained a value of 0.8. The expression of tPRAI then was induced by adding IPTG to a final concentration of 1 mM and allowed to grow for another 5 h until OD₆₀₀ attained 1.7–2.0. Cells were harvested in a flow-through centrifuge (Cepa Z 41G, Lahr, Germany), washed to remove residual culture medium with 50 mM potassium phosphate buffer, pH 7.8, containing 300 mM NaCl, and centrifuged again (Sorvall GSA, 12,000 rpm, 20 min, 4 °C). About 2.6 g cells (wet weight) were obtained per 1 L culture.

The cells were resuspended (2 mL buffer for 1 g wet mass) in 100 mM potassium phosphate buffer, pH 7.8, containing 2 mM EDTA, 1 mM DTT, and 0.3 mM phenylmethylsulfonylfluoride, and lysed by sonification (Branson Sonifier 250, 3×1 min, 50% pulse, 0 °C). The resulting homogenate was centrifuged (Sorvall GSA, 12,000 rpm, 60 min, 4 °C), and about equal amounts of tPRAI were found in the pellet and in the supernatant (Fig. 1). The soluble fraction was assayed for PRAI activity as described by Kirschner et al. (1987). The resulting pellet was resuspended and centrifuged three more times as described. After each centrifugation, the supernatant contained 20–30% of the PRAI activity of the first supernatant. This finding indicates that the insoluble fraction contains tPRAI in a poorly soluble, native-like state. The combined supernatants were heated to 80 °C for 10 min to remove most host proteins. The suspension was centrifuged (Sorvall GSA, 12,000 rpm, 20 min, 4 °C), and the pellet was discarded. The supernatant was loaded on a column (3.6 $\times$ 26 cm) of DEAE-Sepharose fast-flow (Pharmacia) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 0.4 mM DTT, at 4 °C. After washing the column with one volume of equilibration buffer, bound proteins were eluted with a linear gradient from 10 mM (700 mL) to 200 mM (700 mL) potassium phosphate, pH 7.5, containing 2 mM EDTA, 0.4 mM DTT. tPRAI eluted at approximately 70 mM potassium phosphate, as judged from conductivity measurements. Fractions with the highest specific PRAI activity were pooled and concentrated, using PM 10 membranes (Amicon). The concentrated pool was loaded on a column (2.5 $\times$ 90 cm) of Sephacryl S-200 (Pharmacia) that had been equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 300 mM NaCl, 1 mM EDTA, 0.4 mM DTT, at 4 °C. The main UV-absorbing peak contained tPRAI with a purity above 95%. The yield of the purification was approximately 0.47 mg tPRAI from 1 g wet cells (Table 1), corresponding to 0.4-L cell culture. Concentration of this peak to 12–16 mg/mL and overnight storage on ice gave needle-shaped crystals of tPRAI with a purity of more

than 99% (Fig. 1). ePRAI was expressed and purified as described by Eberhard et al. (1995).

Crystallization

Crystallization was performed using the hanging drop vapor diffusion method. The purified protein (11 mg/mL in 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.4 mM DTT) was concentrated to 19.4 mg/mL by ultrafiltration (Centricon-10 centrifugal concentrators, Amicon). Two microliters of the concentrated protein solution was mixed with 2 μ L reservoir solution containing 1.5–2.1 M ammonium sulphate and 10 mM CoCl₂ in 0.1 M MES buffer, pH 6.5. At 4 °C, a few small crystals appeared after about 1 week. To obtain crystals of sufficient size for diffraction experiments, the crystallization setup was subsequently transferred to room temperature, where the crystals grew to the size of $0.2 \times 0.2 \times 1.5$ mm³ after two weeks. The crystals were mounted in glass capillaries and analyzed using a MAR-Research imaging plate area detector mounted on an Elliott GX-20 rotating anode generator (CuK α , 40 kV and 50 mA). Diffraction data evaluation was performed using XDS (Kabsch, 1988).

Analytical methods

Purification of the tPRAI was checked by electrophoresis on 12.5% SDS polyacrylamide gels using the system of Lämmli (1970) and staining with Coomassie blue. The following proteins were used as molecular markers: bovine pancreatic trypsin inhibitor (6.5 kDa), horse heart myoglobin (17.0 kDa), ePRAI (21.1 kDa), α subunit of tryptophan synthase from *E. coli* (28.7 kDa), eIGPS (31.0 kDa), β subunit of tryptophan synthase from *E. coli* (43.0 kDa), bovine serum albumin (66.3 kDa), and rabbit muscle phosphorylase (97.4 kDa). Operational protein concentrations were determined according to Bradford (1976), using bovine serum albumin as the standard.

Sedimentation equilibrium centrifugation of tPRAI was performed at 20 °C with a Beckman analytical ultracentrifuge (model Optima XL A). The tPRAI concentration was 26 μ M in 50 mM potassium phosphate at pH 3.2, pH 4.7, or pH 7.0, containing 0.2 mM EDTA and 0.2 mM DTT. The runs were performed at 18,000 rpm, and the absorption was followed at 278 nm. An assumed partial specific volume of 0.73 mL/g was used for determining the molecular weight. Sedimentation equilibrium runs with tPRT and a mixture of tPRAI and tPRT were performed at 14,000 rpm at 20 °C as described above. Each protein was present at a concentration of 5 μ M in 50 mM Tris/HCl at pH 7.5 containing 100 mM NaCl, 2 mM K₂MgEDTA, and 2 mM DTT.

Absorption spectra were recorded at 22 °C using a diode array spectrophotometer (Hewlett Packard model 8452 A) that was interfaced to a HP Vectra ES/12 computer. CD spectra were monitored with a JASCO model J-720 spectropolarimeter, which was connected to a Philips SX computer. Fluorescence spectroscopy was performed with a Hitachi F-4500 spectrofluorimeter interfaced to a microcomputer (Twix 88-XT personal computer).

The molar absorption coefficient for dimeric tPRAI was determined by second-derivative spectroscopy according to Levine and Federici (1982). A solution of tPRAI (250 μ L in 50 mM potassium phosphate, pH 7.0, 300 mM NaCl) was diluted with 750 μ L of the same buffer in the presence or absence of 7.9 M guanidinium chloride. To determine the molar extinction coefficient under native conditions, the absorption spectrum was normalized by the measured molar concentration of denatured tPRAI chains.

Steady-state kinetics and ligand binding

Progress curves of the essentially irreversible PRAI reaction were recorded fluorimetrically, as described by Hommel et al. (1995). The measurements were performed in 50 mM Tris/HCl buffer, containing 4 mM K₂MgEDTA, 2 mM DTT, with enzyme concentrations between 0.2 nM and 2.5 nM for dimeric tPRAI, and 15 nM for ePRAI. The pH was adjusted at 25 °C to a value that corresponded to a value of 7.5 at the respective temperature, assuming a dpK/dT for Tris buffer of -0.028 (Stoll & Blanchard, 1990). PRA was synthesized at 25 °C with anthranilate phosphoribosyltransferase from yeast (Hommel et al., 1989), at higher temperatures with the thermostable tPRT from *T. maritima* (a gift from A. Ivens; Sterner et al., 1995). Due to the thermolability of PRA (Creighton, 1968), a 30-fold molar excess of PRPP over anthranilic acid was used at 25 °C, 37 °C, and at 45 °C, and a 200-fold molar excess was necessary at 60 °C to guarantee a constant level of substrate. Independent experiments showed that PRPP at the applied concentrations did not affect the shape of the progress curves. Depending on temperature, product inhibition by CdRP was abolished by adding an excess either of eIGPS (Eberhard et al., 1995), or of thermostable IGPS from *Sulfolobus solfataricus* (Hennig et al., 1995). Analysis of the progress curves with the integrated form of the steady-state rate equation was performed as described by Hommel et al. (1995).

The reversible binding of the competitive inhibitor rCdRP to dimeric tPRAI (1.07 μ M) was measured fluorimetrically at 20 °C in 50 mM potassium phosphate buffer containing 0.2 mM EDTA and 0.2 mM EDTA at both pH 7.0 and pH 3.2. Analysis of the binding was performed as described for ePRAI (Eberhard et al., 1995).

Stability measurements of tPRAI and PRA

The half lives of irreversible heat inactivation of both tPRAI and ePRAI were used as an operational criterion of thermostability. The protein concentrations were between 0.25 μ M and 0.7 μ M for dimeric tPRAI and 2.0 μ M for ePRAI in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM K₂MgEDTA and 1 mM DTT. Samples taken after certain time intervals were quenched on ice. Subsequently, the residual activities were measured at 25 °C by recording entire progress curves (see above). Control experiments showed that the course of the progress curves was independent of the incubation time on ice (or room temperature), indicating the complete irreversibility of heat inactivation at the applied temperatures. Furthermore, the analysis of the progress curves showed that K_M^{PRA} of the remaining enzyme was unchanged after the heat treatment, which confirms that it is identical to the native enzyme.

Proteolytic stability was tested by treating tPRAI with different proteases. To this end, tPRAI was dialyzed at 4 °C against 100 mM Tris/acetate buffer at pH 7.8, containing 2 mM EDTA and 1 mM DTT. Aliquots (300 μ L at 1.3 mg/mL) were incubated under different conditions: (1) with 5 μ L of either trypsin (1 mg/mL), 5 μ L ArgC protease (30 U/mL), 5 μ L GluC protease (0.2 mg/mL), or 10 μ L LysC protease (1 U/mL), either at 22 °C (for 10, 60, and 180 min) or at 50 °C (for 60 min); (2) with 5 μ L trypsin (1 mg/mL) or 5 μ L GluC (0.2 mg/mL) in the presence of 0.5% SDS, either at 37 °C (for 90 min) or at 50 °C (for 10–180 min). Proteolysis was stopped by mixing 50 μ L of the reaction mixture with two volumes of sample buffer (Lämmli, 1970). This solution was heated to 95 °C for 10 min and analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels.

To test proteolytic stability under acidic conditions, tPRAI was diluted into 50 mM potassium phosphate at pH 3.2. A sample (180 μ L with 0.22 mg/mL) was incubated at 22 °C with 3 μ L of pepsin (1 mg/mL) for 5–90 min. Proteolysis was stopped by mixing 25 μ L of the reaction mixture with two volumes of sample buffer and 5 μ L of 1 M Tris-base. Analysis on SDS polyacrylamide gels was performed as described above.

The stability of PRA at 25 °C, 37 °C, 50 °C, and 60 °C was measured in 50 mM potassium phosphate buffer, pH 7.5, containing 4 mM K₂MgEDTA. To this end, PRPP and a twofold excess of anthranilic acid were converted to PRA, using either yPRT (Hommel et al., 1989) or tPRT (a gift from A. Ivens). The spontaneous hydrolysis of PRA to anthranilic acid and ribose-5-phosphate (Hommel et al., 1989) is a single exponential process, which was followed by an increase in the fluorescence intensity (excitation at 310 nm, emission at 400 nm). The half life of PRA at 80 °C was extrapolated using an Arrhenius plot.

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