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Properties of tryptophan indole-lyase from a piezophilic bacterium, *Photobacterium profundum* SS9

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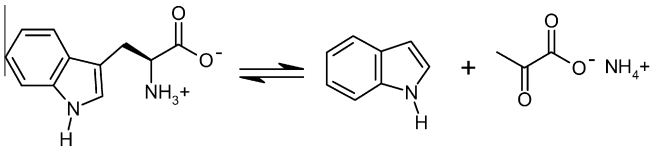
ABSTRACT

Tryptophan indole-lyase (Trpase), PBPRA2532, from *Photobacterium profundum* SS9, a piezophilic marine bacterium, has been cloned, expressed in *Escherichia coli*, and purified. The *P. profundum* Trpase (PpTrpase) exhibits similar substrate specificity as the enzyme from *E. coli* (EcTrpase). PpTrpase has an optimum temperature for activity at about 30 °C, compared with 53 °C for EcTrpase, and loses activity rapidly ($t_{1/2} \sim 30$ min) when incubated at 50 °C, while EcTrpase is stable up to 65 °C. PpTrpase retains complete activity when incubated more than 3 h at 0 °C, while EcTrpase has only about 20% remaining activity. Under hydrostatic pressure, PpTrpase remains fully active up to 100 MPa (986 atm), while EcTrpase exhibits only about 10% activity at 100 MPa. PpTrpase forms external aldimine and quinonoid intermediates in stopped-flow experiments with L-Trp, *S*-Et-L-Cys, *S*-benzyl-L-Cys, oxindolyl-L-Ala, L-Ala and L-Met, similar to EcTrpase. However, with L-Trp a *gem*-diamine is observed that decays to a quinonoid complex. An aminoacrylate is observed with L-Trp in the presence of benzimidazole, as was seen previously with EcTrpase [28] but not with *S*-Et-L-Cys. The results show that PpTrpase is adapted for optimal activity in the low temperature, high pressure marine environment.

Introduction

Tryptophan indole-lyase (Trpase[1], tryptophanase, EC 1.4.99.1) is a pyridoxal-5′-phosphate (PLP) dependent enzyme found in a wide range of bacteria, especially in the enterobacteria, such as *Escherichia coli*, *Vibrio cholerae*, and *Proteus vulgaris* [1]. Trpase catalyzes the reversible hydrolytic carbon–carbon bond cleavage reaction of L-Trp to give indole and ammonium pyruvate (Eq. (1)). Indole



(1)

has long been considered as simply a waste product of bacteria metabolism of proteins, but recent work has found that indole is a chemical signal for bacteria, regulating gene expression [2] and a wide variety of physiological processes, including biofilm formation [3,4] and plasmid replication [5]. Thus, the expression of Trpase is stringently regulated in *E. coli*, with induction by high concentrations of L-Trp [6], but repression in the presence of glucose [7]. Recently, the genome of a piezophilic and psychrophilic marine bacterium, *Photobacterium profundum* SS9, was sequenced, and it was found to contain 3 different copies of *tna*A, the gene coding for Trpase [8], although *E. coli* only contains a single copy of *tna*A. *P. profundum* SS9 has an optimal growth temperature of 15 °C and pressure of 28 MPa, and can grow at pressures up to 90 MPa [8]. A number of genes were found to be selectively expressed in *P. profundum* SS9 grown at 28 MPa compared to growth at 0.1 MPa (0.986 atm), including one of the *tna*As, PBPRA2532 [8]. In this work, we have cloned, expressed, and purified the PBPRA2532 Trpase from *P. profundum* (PpTrpase), and we have compared its properties with the *E. coli* enzyme (EcTrpase).

Experimental methods

Materials

S-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as previously described [9]. Lactate dehydrogenase (LDH) from rabbit muscle, L-tryptophan, *S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, *S*-benzyl-L-cysteine, PLP, L-methionine and NADH were purchased from

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[1] *Abbreviations used:* PLP, pyridoxal-5′-phosphate; Trpase, tryptophan indole-lyase (tryptophanase) [EC 4.1.99.1]; EcTrpase, Trpase from *Escherichia coli*; PpTrpase, Trpase from *Photobacterium profundum* SS9 PBPRA2532; SOPC, *S*-(*o*-nitrophenyl-L-cysteine).

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United States Biochemical Corp. (USB). β-Cl-L-Alanine hydrochloride was prepared from L-serine as described [10]. α-[^{2}H]-L-Trp was prepared as previously described [11].

Cloning and expression of P. profundum Trpase

Forward and reverse primers based on the DNA sequence of *P. profundum* SS9 *tna*A (PBPRA2532) were designed, and PCR was performed with 1 ng of genomic DNA. The primers used were 5′-CACCATGGAAAACTTTAAACACTTAC-3′ for the forward primer and 5′-GATCGTGATTAAGCTTCTAGAATC-3′ for the reverse primer. The PCR product was cloned with pET-100/D-TOPO (Invitrogen). Plasmids were isolated and sequenced to confirm the presence and correct sequence of the *P. profundum* PBPRA2532 gene. The plasmid pET100-PBPRA2532 was then used to transform *E. coli* BL21(DE3) *tn*5:*tna*A [12] for expression of PpTrpase without contamination by EcTrpase.

An overnight culture of *E. coli* BL21(DE3) *tn*5:*tna*A pET100-PBPRA2532 was grown in 5 mL of LB medium containing 35 μg/mL of kanamycin sulfate and 100 μg/mL of ampicillin. The overnight culture was used to inoculate 1 L of LB medium containing 35 μg/mL of kanamycin sulfate and 100 μg/mL of ampicillin, and the culture was grown with shaking at 37 °C in a water bath until the OD_{600} was 0.6–0.8. The temperature of the bath was then rapidly reduced to 20 °C by addition of ice, 0.1 mM IPTG was added to the culture, and the cell suspension was shaken at 20 °C for 18 h. The cells were then collected by centrifugation for 20 min at 10,000*g*, suspended in 30 mL 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM 2-mercaptoethanol, 50 μM PLP, and frozen at −78 °C until used for enzyme purification.

Enzyme purification

EcTrpase was purified by hydrophobic chromatography on a column of CL-Sepharose 4B, as described previously [13], except that a gradient of $(NH_4)_2SO_4$ from 40% to 20% saturation was used for elution, rather than a stepwise elution. For PpTrpase, a column of Phenyl-Sepharose CL-4B was used, since in contrast to EcTrpase, the enzyme was not retained on a column of Sepharose CL-4B in 40% saturated $(NH_4)_2SO_4$. The crude extract and protamine treatment was performed as described previously [13], and then the solution was brought to 20% saturation by addition of solid ammonium sulfate. After loading, the column was washed with 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM 2-mercaptoethanol, 50 μM PLP, containing 20% ammonium sulfate until the absorbance returned to baseline, then it was eluted with a gradient of decreasing ammonium sulfate. Peak fractions were pooled and frozen at −78 °C. Protein was determined by the method of Bradford [14], with purified wild-type EcTrpase as a standard. Enzyme activity during purification was routinely measured with 0.6 mM *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) in 50 mM potassium phosphate, pH 8.0, 50 μM PLP, at 25 °C [15], following the decrease in absorbance at 370 nm ($\Delta\varepsilon = -1.86 \times 10^3\ M^{-1}\ cm^{-1}$) using a Cary 1E UV/vis spectrophotometer equipped with a 6 × 6 Peltier temperature controlled cell compartment. Enzyme activity with L-Trp, *S*-alkyl-L-cysteines, and β-chloro-L-Ala were performed in the same buffer using the lactate dehydrogenase coupled assay [16], with 0.1 mM NADH and 20 μg/mL lactate dehydrogenase, measuring the absorbance decrease at 340 nm ($\Delta\varepsilon = -6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

Temperature dependence of Trpase reactions

The temperature dependence of the reactions of PpTrpase and EcTrpase was performed with SOPC in 50 mM KPi, pH 8.0, 50 μM PLP. For the temperature dependence of the activity, the enzyme was added last to the reaction mixtures pre-equilibrated at the desired temperature, and the immediate decrease in absorbance at 370 nm was measured. For the temperature stability measurements, the enzymes were incubated at 0 °C on ice, or at 50 °C in a water bath, and aliquots were removed at various times and assayed with SOPC at 25 °C.

Stopped-flow reactions

Stopped-flow experiments were carried out at ambient temperature (ca. 22–23 °C) using an RSM-1000 instrument from OLIS, Inc. (Bogart, Georgia, USA), equipped with a stopped-flow mixing chamber, as described previously [17,18]. The stopped-flow mixer has a 10 mm path length and a dead time of less than 2 ms. Absorbance spectra were collected over the wavelength range from 240 to 800 nm at a rate of 1000 scans s^{-1}. Enzymes for stopped-flow measurements were passed through a PD-10 gel filtration column equilibrated with 0.05 M potassium phosphate, pH 8.0, to remove excess PLP, immediately prior to use.

Enzyme activity and absorption spectra under hydrostatic pressure

The effects of hydrostatic pressure on the rates and absorption spectra were measured using a Cary 14 UV/Vis spectrophotometer modified by OLIS, Inc. to contain a high pressure cell from ISS (Champaign, Illinois, USA), equipped with a manual pressure pump from High Pressure Equipment Co., using spectroscopic grade ethanol as the pressurizing fluid. The cell was maintained at 25 °C with an external circulating water bath. The enzyme solutions were contained in 1 mL quartz bottles with a 9 mm pathlength, capped with Teflon tubing. A buffer blank at 1 bar was used to obtain a baseline reading. The buffer, triethanolamine hydrochloride, pH 8.0, was chosen since the pK_a is 7.88 and the ΔV_0 for ionization is 4.5 ± 0.3 mL/mol [19], so the pressure change will not significantly change the pH. The pressure-dependent activity was measured at 25 °C in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 100 mM L-serine and 250 μM indole, measuring the synthesis of tryptophan at 290 nm ($\varepsilon = 1800\ M^{-1}\ cm^{-1}$) [20], in order to avoid potential problems with the lactate dehydrogenase coupling reaction under pressure.

Data analysis

The rapid-scanning stopped-flow data were analyzed by global analysis of all spectra at all wavelengths using the Globalworks program provided by OLIS [21]. The spectra were fitted to the minimum number of species and exponential processes to adequately describe the data based on residuals and standard deviation, using Eq. (2), where A_t is the absorbance at a wavelength at time t, A_i is the absorbance at that wavelength for each phase, k_i is the rate constant for each phase, and A_0 is the baseline absorbance at that wavelength of the reaction mixture. The effects of hydrostatic

$$A_t = \sum A_i * \exp(-k_i * t) + A_0 \quad (2)$$

pressure on the absorption spectra were analyzed by global analysis using the Globalworks program, as previously described [17,18]. The absorption spectra were corrected for solvent compressibility using a modified Tait equation [22]. The pressure dependent changes at a single wavelength were fitted to a Boltzmann function, in Eq. (3), where A_p is the observed absorbance at pressure P, ΔA is the pressure-dependent absorbance change, K_{eq} is the pressure independent value of the equilibrium constant, ΔV is the reaction volume change, and A_∞ is the absorbance background at infinite pressure.

$$A_p = \Delta A(K_{eq} * \exp(-P * \Delta V))/(1 + K_{eq} * \exp(-P * \Delta V)) + A_\infty \quad (3)$$

Results

Expression and purification of P. profundum Trpase

It was found that induction of the *P. profundum tna*A PBPRA2532 gene at 37 °C did not result in significant activity in cell extracts, but large amounts of insoluble inclusion bodies were seen in the pellets after cell disruption. However, induction with IPTG followed by incubation overnight at 20 °C provided very high expression of soluble, active Trpase. The expression was performed in *E. coli* BL21(DE3)*tn*5:*tna*A with a transposon in the genomic *tna*A to avoid contamination by the *E. coli* enzyme. In contrast to the *E. coli* enzyme, PpTrpase did not adsorb to a column of Sepharose CL-4B in 40% saturated ammonium sulfate [13], so the purification was performed on a Phenyl-Sepharose CL-4B column, with adsorption in 20% ammonium sulfate saturated buffer and elution with a linear gradient of buffer containing no ammonium sulfate. The purified Trpase was >95% pure by PAGE and exhibited a specific activity of 10 U/mg with SOPC at 25 °C, compared to the specific activity of EcTrpase, about 34 U/mg [13].

Substrate specificity of P. profundum Trpase

PpTrpase has high activity for β-elimination of L-Trp, SOPC, and β-Cl-L-Ala, but values of k_{cat} and k_{cat}/K_m are 3- to 5-fold lower than the *E. coli* enzyme (Table 1). It is particularly interesting that *S*-alkyl-cysteines with small substituents have at least 10-fold lower values of k_{cat} for PpTrpase (Table 1) than for EcTrpase [11,13,23] .

Effect of temperature on P. profundum Trpase activity

The optimal temperature for PpTrpase activity with SOPC is 30 °C, compared with an optimum greater than 50 °C with EcTrpase (Fig. 1). Incubation of PpTrpase at 50 °C result in rapid loss of activity, while EcTrpase is considerably more stable on incubation (Fig. 2). In contrast, when EcTrpase is incubated at 0 °C, it loses activity rapidly, as seen in Fig. 3 [24,25]. However, PpTrpase shows little change in activity on incubation at 0 °C (Fig. 3).

Effect of pressure on P. profundum Trpase

The effect of increasing hydrostatic pressure on the specific activity of PpTrpase and EcTrpase was determined (Fig. 4). The activity of EcTrpase decreases at pressures above 50 MPa, and by 100 MPa is less than 10% of the activity at 1 bar. When the samples incubated at 100 MPa were decompressed to 1 bar, activity was immediately recovered to about 75% that of samples measured at 1 bar. In contrast, PpTrpase has greater than 90% activity remaining at 100 MPa (Fig. 4). It is interesting that both enzymes show an initial increase in activity with pressure, reaching a maximum of about 140% at 30 MPa for EcTrpase and 60 MPa for PpTrpase.

Table 1
Substrate specificity of *P. profundum* and *E. coli* Trpase.[a]

Substrate	*P. profundum*		*E. coli*	
	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
L-Trp	2.5 ± 0.10	(7.6 ± 0.4) × 10^3	6.8[b]	3.0 × 10^4 [b]
SOPC	13.9 ± 4.2	(2.9 ± 0.3) × 10^4	44[b]	5.1 × 10^5 [b]
S-Me-L-Cys	0.2 ± 0.07	60 ± 50	5.0[c]	330 [c]
S-Et-L-Cys	0.4 ± 0.05	280 ± 130	6.0[d]	9.0 × 10^3 [d]
S-Bzl-L-Cys	1.1 ± 0.16	(1.1 ± 0.7) × 10^4	5.2 ± 0.4[e]	(8.1 ± 0.3) × 10^4 [e]
β-Cl-L-Ala	3.0 ± 0.10	770 ± 70	12.4 ± 0.6	(2.4 ± 0.1) × 10^3

[a] The reactions were performed in 50 mM potassium phosphate, pH 8.0, with 50 μM PLP, at 25 °C.
[b] From Ref. [13].
[c] From Ref. [11].
[d] From Ref. [12].
[e] From Ref. [23].

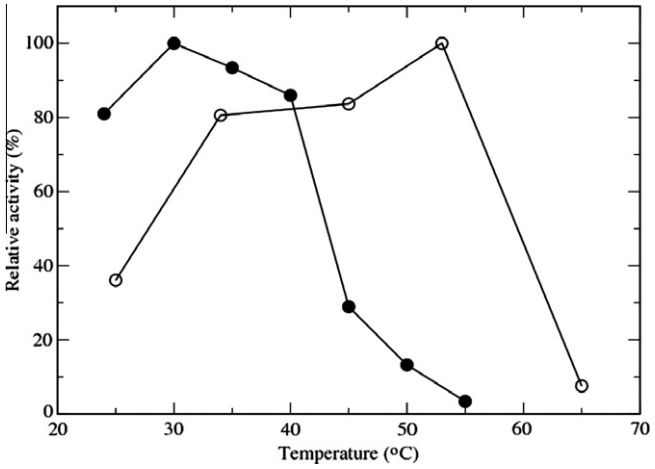


Fig. 1. Effect of temperature on activity of Trpases from *E. coli* and *P. profundum*. Open circles: EcTrpase; closed circles, PpTrpase.

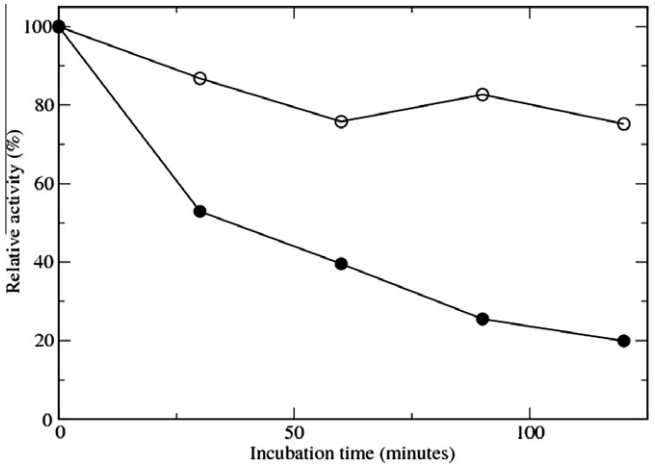


Fig. 2. Effect of incubation at 50 °C on activity of Trpases from *E. coli* and *P. profundum*. Open circles: EcTrpase; closed circles, PpTrpase.

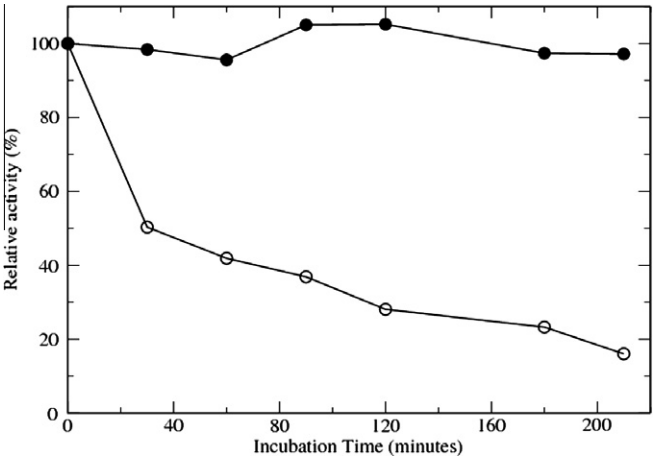


Fig. 3. Effect of incubation at 0 °C on activity of Trpases from *E. coli* and *P. profundum*. Open circles: EcTrpase; closed circles, PpTrpase.

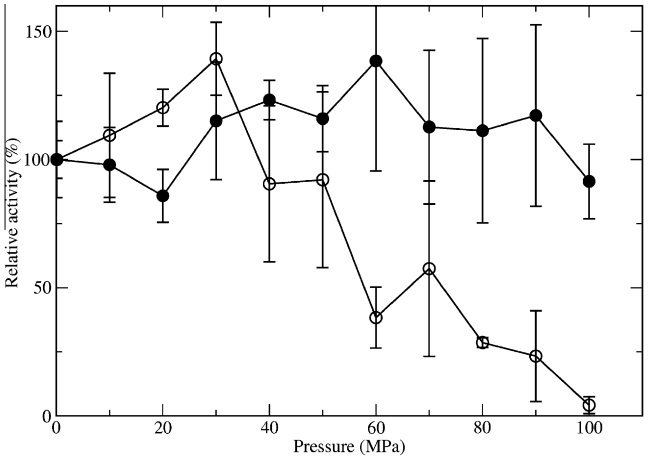


Fig. 4. Effect of hydrostatic pressure at 25 °C on activity of Trpases from *E. coli* and *P. profundum*. Open circles: EcTrpase; closed circles, PpTrpase.

EcTrpase exhibits absorption peaks at about 340 and 420 nm, corresponding to two different forms of the PLP cofactor [26,27]. Application of hydrostatic pressure decreases the 420 nm band and increases the 340 nm band for EcTrpase [25], consistent with a conformational change coupled with the change in spectrum. PpTrpase exhibits a very similar absorption spectrum, and similar effects of increasing pressure (Fig. 5). The absorption changes at 420 nm are fully reversible upon decompression, as can be seen by comparing the open circles (compression) and the filled circles (decompression) in the inset Fig. 5. Fitting the spectra in Fig. 5 provides $\Delta V = -39 \pm 7$ mL/mol and $K_{eq} = 0.106 \pm 0.016$, compared to $\Delta V = -38 \pm 3$ mL/mol and $K_{eq} = 0.65 \pm 0.03$ for EcTrpase [25]. The dashed lines are the calculated spectra from the global analysis. The solid line in the inset is the calculated curve using Eq. (3) and the parameters above.

Stopped-flow kinetics of P. profundum Trpase

Mixing of PpTrpase with 10 mM L-Trp in the stopped-flow spectrophotometer results in formation of intermediates absorbing at about 340, 420 and 505 nm (Fig. 6A), similar to what is seen with EcTrpase [28–30]. There is an initial rapid increase at about 340 nm and decrease at 420 nm, together with a small increase at 505 nm, followed by a decrease at 340 nm as the 420 and 505 nm peaks increase (Fig. 4B), and finally a slow increase at 505 nm, with $1/\tau_1 = 230 \pm 22$ s^{-1}, $1/\tau_2 = 10.2 \pm 1.3$ s^{-1} and $1/\tau_3 = 1.36 \pm 0.20$ s^{-1}. When the experiment was performed with α-[^{2}H]-L-Trp, the rate constants were $1/\tau_1 = 95 \pm 10$ s^{-1}, $1/\tau_2 = 12.2 \pm 1.7$ s^{-1} and $1/\tau_3 = 0.92 \pm 0.20$ s^{-1} (data not shown). Thus, there is a small kinetic isotope effect of 2.4 on the fast phase of the reaction. The decay of the 340 peak and concomitant increase at 420 and 505 nm do not show an isotope effect. When 5 mM benzimidazole is included in the reactions with tryptophan, after an initial increase at 505 nm, the 420 and 505 nm peaks decay concomitant with the formation of a strong peak at about 350 nm (Fig. 4C and D), with $1/\tau_1 = 260 \pm 22$ s^{-1}, $1/\tau_2 = 20.0 \pm 2.3$ s^{-1} and $1/\tau_3 = 0.69 \pm 0.06$ s^{-1}. This is very similar to what was seen with EcTrpase [28–30].

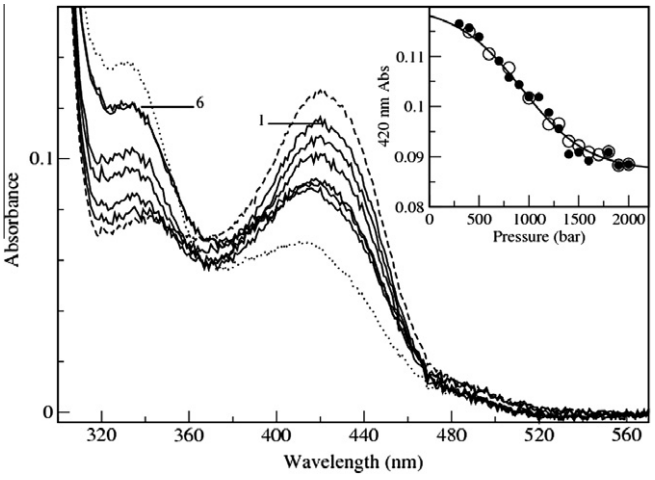


Fig. 5. Effect of hydrostatic pressure at 25 °C on the absorption spectrum of PpTrpase. Curve 1, 300 bar; Curve 2, 700 bar; Curve 3, 1000 bar; Curve 4, 1400 bar; Curve 5, 1800 bar; Curve 6, 2000 bar. The dashed and dotted curves are the fitted spectra of the two components at 0 and infinite pressure, respectively. Inset: Absorbance at 420 nm as a function of pressure. Filled circles, compression; open circles, decompression. The line is the calculated curve using Eq. (3) with the parameters given in the text.

When EcTrpase is mixed with substrates and inhibitors such as L-Met, *S*-Et-L-Cys, *S*-benzyl-L-Cys, oxindoly-L-Ala or L-Ala in the stopped-flow instrument, strongly absorbing quinonoid intermediates with λ_{max} about 500 nm are formed [28,31]. We observed similar results with PpTrpase. It is particularly interesting that *S*-Et-L-Cys rapidly forms a quinonoid intermediate at 508 nm, as can be seen in Fig. 7, with k_{obs} of 18 s^{-1}, very similar to EcTrpase [28], even though the k_{cat} value with this substrate is much slower than that for EcTrpase (Table 1). Furthermore, when the stopped-flow experiment with *S*-Et-L-Cys is performed with PpTrpase in the presence of 5 mM benzimidazole, there is no observed decay of the quinonoid intermediate to the 350 nm band of the aminoacrylate (data not shown), while EcTrpase forms an aminoacrylate intermediate from *S*-Et-L-Cys in the presence of benzimidazole [28–31]. The rapid-scanning stopped-flow spectra for the reactions of L-Met, L-Ala, *S*-benzyl-L-Cys and oxindolyl-L-Ala with PpTrpase are included in the Supplementary data.

Discussion

There has been considerable interest in the properties of enzymes from organisms adapted to extreme environments in recent

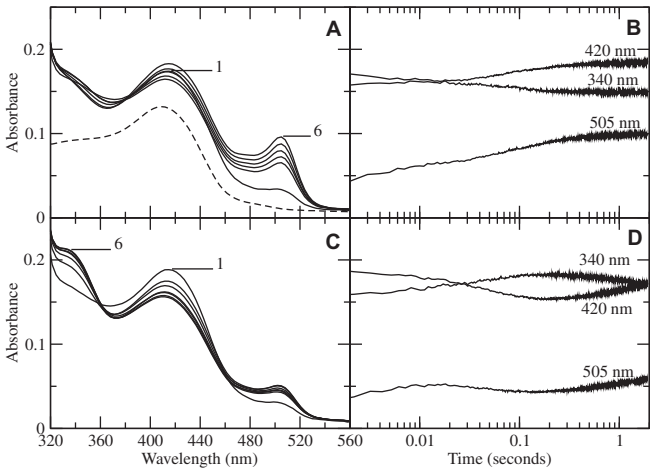


Fig. 6. Reaction of PpTrpase and L-Trp. (A) Rapid-scanning stopped-flow spectra for the reaction of 1.5 mg/mL PpTrpase with 10 mM L-Trp in 0.02 M KPi, pH 8.0, 0.16 M KCl. Scans are shown at 0 (Curve 1), 0.020 (Curve 2), 0.04 (Curve 3), 0.08 (Curve 4), 0.32 (Curve 5) and 0.64 (Curve 6) seconds after mixing. (B) Time courses for the reaction in A at 340, 420 and 505 nm. (C) Rapid-scanning stopped-flow spectra for the reaction of 1.5 mg/mL PpTrpase with 10 mM L-Trp and 5 mM benzimidazole in 0.02 M KPi, pH 8.0, 0.16 M KCl. Scans are shown at 0 (Curve 1), 0.020 (Curve 2), 0.04 (Curve 3), 0.08 (Curve 4), 0.32 (Curve 5) and 0.64 (Curve 6) seconds after mixing. The dashed line is the spectrum of the enzyme alone. (D) Time courses for the reaction in C at 340, 420 and 505 nm.

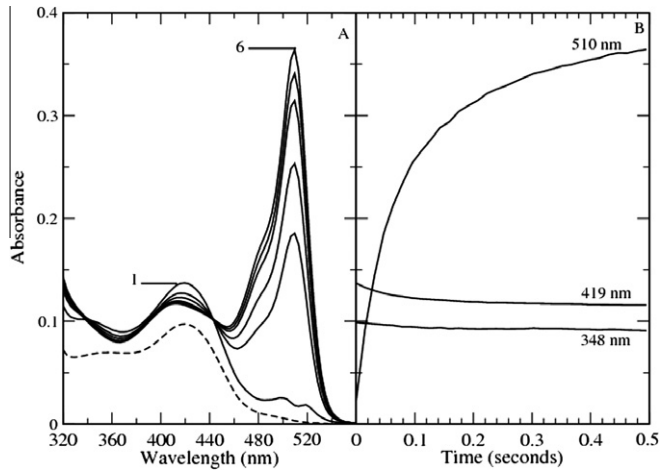


Fig. 7. Reaction of PpTrpase and *S*-Et-L-Cys. (A) Rapid-scanning stopped-flow spectra for the reaction of 1 mg/mL PpTrpase with 20 mM *S*-Et-L-Cys in 0.02 M KPi, pH 8.0, 0.16 M KCl. Scans are shown at 0.001 (Curve 1), 0.047 (Curve 2), 0.095 (Curve 3), 0.207 (Curve 4), 0.393 (Curve 5) and 0.495 (Curve 6) seconds after mixing. The dashed line is the spectrum of the enzyme alone. (B) Time courses for the reaction in A at 340, 420 and 505 nm.

years. Most of these studies have focused on enzymes from thermophilic microorganisms, adapted to living at high temperatures in terrestrial hot springs or around marine thermal vents. In the case of Trpase, the enzymes from a symbiotic thermophile, *Symbiobacterium thermophilum*, have been cloned, expressed and purified [32]. In contrast, there has been much less work done with psychrophilic and piezophilic enzymes. We were interested in the Trpases from *P. profundum* SS9, since this organism is adapted to both high pressure (pressure optimum 10–30 MPa, growth up to 90 MPa) and low temperature growth, with an optimum of 15 °C. In particular, one of the three Trpases in the genome of *P. profundum* SS9, PBPRA2532, is preferentially expressed when cells are grown at 28 MPa [8]. Thus, it seemed likely to us that this enzyme would show an adaptation to the high pressure and low temperature environment that would be reflected in its physical properties, so we cloned, expressed and purified it and compared it with the *E. coli* enzyme.

Physical properties

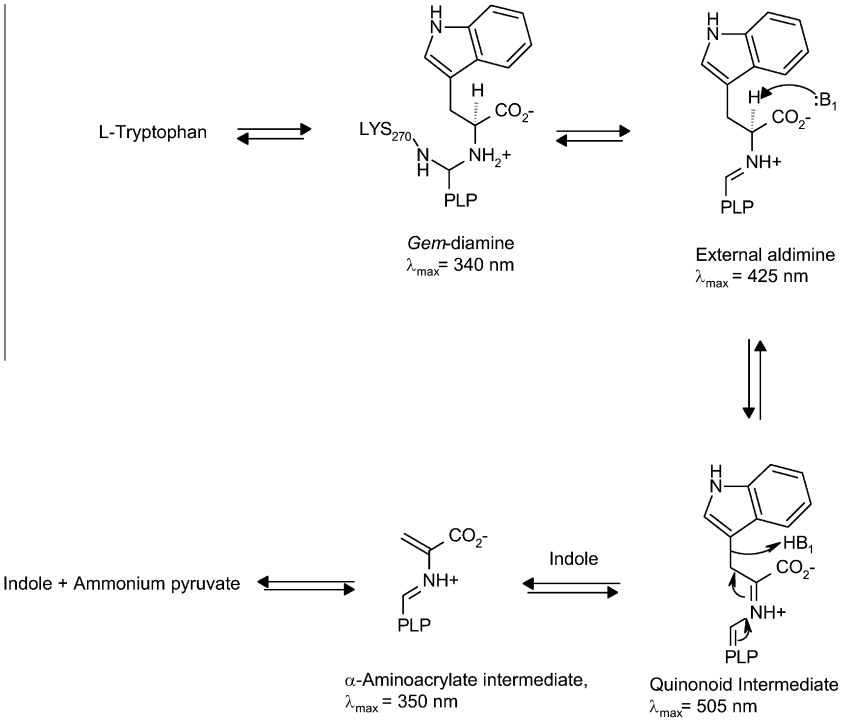
The well-characterized EcTrpase is relatively thermostable. Indeed, a heat treatment for 15 min at 65 °C is one of the steps in the classic purification procedure [16]. The temperature optimum for EcTrpase is between 50 and 60 °C (Fig. 1), whereas PpTrpase shows a sharp decrease in activity above 40 °C. This decrease is probably due to denaturation, since the PpTrpase loses activity rapidly when incubated at temperatures above 40 °C (Fig. 2). Thus, PpTrpase is clearly adapted for the low temperatures (4–15 °C) at which the organism grows. This is also seen strikingly in the cold inactivation experiment, where EcTrpase rapidly loses activity upon incubation at 0 °C (Fig. 3) [24,25], while PpTrpase retains about 80% activity. This cold lability of EcTrpase has been attributed to facile dissociation of the tetramer into non-catalytic dimers, with concomitant loss of PLP [24,25]. The loss of activity of EcTrpase in the cold is reversible, since activity is restored on incubation at 37 °C for about 1 h [24,25]. Previously, residues Val15, Ile16 and Val59 were identified as forming a hydrophobic core which helps to stabilize the tetramer of EcTrpase [25]. In a sequence alignment, we find that Val59 of EcTrpase is changed to Ile59 in PpTrpase. This Ile residue increases the strength of the hydrophobic interaction in the core and thus may be at least partially responsible for the increased cold stability of PpTrpase shown in Fig. 3.

There are also dramatic differences in the effects of hydrostatic pressure on the activity of EcTrpase and PpTrpase. PpTrpase retained complete activity up to at least 100 MPa (Fig. 4), well above the physiological pressures (28–45 MPa) that *P. profundum* is routinely exposed to in its marine environment. In contrast, EcTrpase exhibits less than 10% activity at 100 MPa (Fig. 4). The loss of activity of EcTrpase under pressure is rapidly reversible, since the activity is immediately restored when the samples are decompressed. This suggests that the pressure-dependent reversible loss of activity is the result of a transition state effect, due to a positive $\Delta V^{\ddagger}$, rather than subunit or cofactor dissociation, since cold-inactivated EcTrpase recovers activity relatively slowly by a second-order reassociation of PLP with the apoenzyme [24,25]. It is interesting that PpTrpase has somewhat lower activity than EcTrpase at 25 °C and 0.1 MPa, but higher activity at pressures above 60 MPa. This may be due to the structural modifications that are required for maintenance of activity under low temperature and high pressure making the enzyme more flexible. The effects of pressure on the spectra of the resting enzymes from *P. profundum* and *E. coli* are very similar (Fig. 5), changing the ratio of intensity of the 420 and 340 nm bands. This change in ratio is associated with a blue shift in the 420 nm band (Fig. 5), suggesting a change in the polarity of the cofactor environment that favors the 340 nm form. The 420 nm peak has been assigned to the ketoenamine tautomer of the internal aldimine [26], while the 340 nm peak has been suggested to be an enolimine tautomer [26] or a nucleophilic adduct [27] of the internal aldimine. The dotted lines in Fig. 5 are the fitted spectra from global analysis. Since both the high and low pressure fitted spectra still contain both peaks, and the volume change is negative, the pressure dependence of the spectra is likely due to a conformational change which alters the environment of the PLP and affects the equilibrium. The ratio of intensity of the 340 and 420 nm peaks is also pH-dependent [13,33,34], with an apparent pK_a of 7.5.

Catalytic properties

PpTrpase has similar, but slightly lower, activities for β-elimination of L-Trp, β-Cl-L-Ala, *S*-benzyl-L-Cys and SOPC (Table 1) compared to EcTrpase. However, the k_{cat} values for *S*-Me and *S*-Et-L-Cys are only 10% that of EcTrpase. Rapid-scanning stopped-flow experiments showed that quinonoid intermediates are formed from these substrates with rate constants similar to the *E. coli* enzyme (Fig. 5). Thus, the slow reaction of these substrates must be due to much slower elimination of the small alkylthiols from the quinonoid complex. This proposal is supported by the lack of formation of an aminoacrylate intermediate absorbing at 350 nm when PpTrpase is mixed with *S*-Et-L-Cys and benzimidazole. Since EcTrpase does form an aminoacrylate intermediate under the same conditions [28], this observation implies that elimination of the alkylthiol is much slower than the release of pyruvate, resulting in very little accumulation of the aminoacrylate intermediate, which binds benzimidazole as an uncompetitive inhibitor [28]. It is possible that proton transfer to the leaving thiolate group is inefficient for these substrates. The k_{cat} value increases for alkyl Cysteines with bulky groups like benzyl (Table 1), suggesting that the binding of large groups can lower the activation energy for elimination.

The reaction of PpTrpase with L-Trp shows the formation of external aldimine and quinonoid intermediates (Scheme 1), absorbing at 420 and 505 nm, similar to EcTrpase [28–30], in rapid-scanning stopped-flow experiments (Fig. 6). The formation of the quinonoid intermediate is biphasic, with the fast phase showing a kinetic isotope effect of 2.4 when α-[^{2}H]-L-Trp is used. This



Scheme 1.

compares to an isotope effect of 3.6 on quinonoid formation with EcTrpase [29,35]. However, an additional intermediate absorbing at 340 nm is seen with PpTrpase (Fig. 6). This intermediate forms rapidly at the same rate as a 505 nm quinonoid intermediate, but then decays to give an equilibrating mixture of external aldimine and quinonoid species (Fig. 6). There is no kinetic isotope effect on the decay of the 340 nm peak when α-[^{2}H]-L-Trp is used. The rapid formation of the 340 nm peak from the internal aldimine and the decay to an external aldimine suggests that the 340 nm intermediate is a *gem*-diamine, the conversion of which to the external aldimine is rate-determining. Thus, the 340 nm intermediate is assigned to a *gem*-diamine complex of L-Trp and PLP (Scheme 1). There seem to be two different forms of the enzyme, one of which reacts rapidly to form external aldimine and quinonoid intermediates, and one which reacts slowly. These enzyme species must be in slow equilibrium. The resting enzyme exists in two forms, which have absorption maxima at 338 and 420 nm (Fig. 5), similar to EcTrpase. It is possible that the interconversion of these two species is slow for PpTrpase but relatively fast for EcTrpase. The reaction of PpTrpase with L-Trp in the presence of benzimidazole shows a transient aminoacrylate peak absorbing at 350 nm (Fig. 6C and D) forming with a rate constant of 18 s^{-1}, similar to the rate constant of 30 s^{-1} for aminoacrylate formation by EcTrpase [29]. Thus, the slower k_{cat} for PpTrpase with L-Trp is not due to slow elimination.

Piezophilic enzymes

In contrast to thermophilic enzymes, there have only been a few piezophilic enzymes described to date. Although no evidence for high pressure adaptation of a dihydrofolate reductase from *Moritella profunda* was found, based on structure and activity comparisons [36], a recent study of several dihydrofolate reductases found evidence for piezophilicity in the enzymes from the piezophiles, *P. profundum* and *Moritella yayanosii*, compared to the enzymes from *E. coli*, and moderate piezophiles, *Shewanella violacea* and *Moritella japonica* [37]. An isopropylmalate dehydrogenase from *Shewanella benthica* DB21MT-2 isolated at a depth of 11,000 m in the Mariana Trench also showed evidence of piezophilicity [38]. The effects of pressure on oligomerization of single-stranded DNA binding proteins from various species of *Shewanella* were examined, and the protein from a piezosensitive strain was found to undergo pressure-dependent dissociation of the tetramer more readily than that from piezotolerant or piezophilic strains [39]. Our data reported herein indicate that the Trpase PBPRA2532 from *P. profundum* SS9 should be included in the list of known piezophilic enzymes.

Conclusions

Although EcTrpase and PpTrpase PBPRA2532 are highly homologous (>80% identity), they exhibit significant differences in physical and catalytic properties. EcTrpase is much more heat-stable, while PpTrpase is more stable to cold and retains higher activity under hydrostatic pressure. Both enzymes catalyze eliminations of a range of β-substituted amino acids, as well as tryptophan, but *S*-alkyl cysteines are much poorer substrates for PpTrpase. Rapid-scanning stopped-flow experiments show that similar intermediates are formed with both enzymes, but a *gem*-diamine is formed with PpTrpase and L-Trp that is not observed with *EcTrpase*. The results show that PpTrpase PBPRA2532 is adapted for optimal activity in the low temperature, high pressure marine environment.

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