Extremophiles (2004) 8:455–462
DOI 10.1007/s00792-004-0406-3

ORIGINAL PAPER

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Extrinsic factors potassium chloride and glycerol induce thermostability in recombinant anthranilate synthase from *Archaeoglobus fulgidus*

Received: 27 July 2003 / Accepted: 1 June 2004 / Published online: 2 July 2004

Abstract Thermostable anthranilate synthase from the marine sulfate-reducing hyperthermophile *Archaeoglobus fulgidus* has been expressed in *Escherichia coli*, purified, and characterized. The functional enzyme is an $\alpha_2\beta_2$ heterotetrameric complex of molecular mass 150 ± 15 kDa. It is composed of two TrpE (50 kDa) and two TrpG (18 kDa) subunits. The extrinsic factors glycerol (25%) and potassium chloride (2 M) stabilized the recombinant enzyme against thermal inactivation. In the presence of these extrinsic factors, the enzyme was highly thermostable, exhibiting a half-life of thermal inactivation of about 1 h at 85°C. The kinetic constants for the enzyme under these conditions were: K_m (chorismate) 84 μM, K_m (glutamine) 7.0 mM, k_{cat} 0.25 s^{-1}, and pH optimum 8.0. The enzyme was competitively, though non-cooperatively, inhibited by tryptophan.

Keywords Anthranilate synthase · Hyperthermophile · Thermostable · Cooperative · Extrinsic factor

Introduction

Enzymes from hyperthermophiles have exceptional thermostability (Jaenicke and Bohm 1998); and, for this reason, they are recognized as having great potential as biocatalysts for industrial processes (Adams et al. 1995). However, hyperthermophiles are often difficult to culture and grow in a laboratory setting because of their unusual growth requirements and the caustic nature of their metabolic processes (Huber et al. 1995; Adams and Kelley 1998). For this reason, it is often of interest to use recombinant DNA technology to express hyperthermophilic enzymes in a heterologous host, such as *Escherichia coli*. This approach, too, is frequently fraught with technical problems, such as low expression levels due to differences in codon usage and low solubility due to differences in protein-folding machinery. Problems of heterologous expression are compounded when the enzyme to be expressed is oligomeric or hetero-oligomeric. Reflecting these challenges, only a handful of hetero-oligomeric proteins from hyperthermophiles so far have been expressed in and purified from *E. coli* (Krah et al. 1997; Buhler et al. 1998; Musfeldt et al. 1999; Knöchel et al. 1999).

Anthranilate synthase catalyzes the first committed step of the pathway by which tryptophan is synthesized from chorismate (Scheme 1). Anthranilate synthases from a number of microorganisms, including the mesophiles *E. coli* (Baker and Crawford 1966), *Salmonella typhimurium* (Tamir and Srinivasan 1969; Henderson et al. 1970; Bauerle et al. 1987), and *Serratia marcescens* (Zalkin and Hwang 1971; Robb et al. 1971) and the hyperthermophile *Solfolobus solfataricus* (Tutino et al. 1997) have been studied in terms of their kinetic and regulatory properties. The crystal structures of anthranilate synthase from *Sol. solfataricus* (Knöchel et al. 1999), *Sal. typhimurium* (Morollo and Eck 2001), and *Ser. marcescens* (Spraggon et al. 2001) have been determined; and the structures apparently reveal two different quaternary arrangements of subunits. The anthranilate synthases from *Sal. typhimurium* (Henderson et al. 1970; Caligiuri and Bauerle 1991a, b) and *Ser. marcescens* (Zalkin and Hwang 1971) are cooperatively regulated by the feedback inhibitor tryptophan, but studies of recombinant *Sol. solfataricus* anthranilate synthase have shown the enzyme to be non-cooperative (Tutino et al. 1997), exhibiting Michaelis–Menten kinetics in the

Communicated by F. Robb

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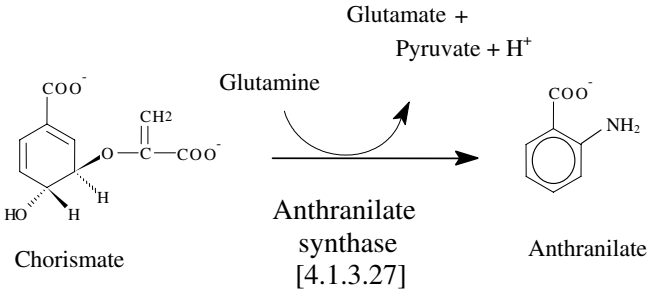

Scheme 1

presence of tryptophan. In *Ser. marcescens* and *Sol. solfataricus*, the enzyme is a heterotetramer of two TrpE and two TrpG subunits (Crawford 1989). The TrpG subunit contains amidotransferase activity, whereas TrpE contains the anthranilate synthase activity of the enzyme. However, in *Sal. typhimurium* and *E. coli*, a bifunctional TrpG(D) subunit replaces TrpG (Grieshaber and Bauerle 1974). (Note that TrpD has phosphoribosyl transferase activity and catalyzes the next step of the pathway.) The TrpE subunit of the *Sal. typhimurium* enzyme can catalyze an ammonia-dependent reaction by itself, but TrpG is required for the glutamine-dependent activity in the pathway by which tryptophan is synthesized from chorismate (Zalkin and Kling 1968). A divalent Mg^{++} ion is required for the catalysis. The tryptophan-binding site of the enzyme is located on the TrpE subunit (Zalkin and Kling 1968) and amino acid residues important for feedback regulation by tryptophan have been identified within *Sal. typhimurium* TrpE (Caligiuri and Bauerle 1991a). Interestingly, a dimeric anthranilate synthase from the hyperthermophile *Thermococcus kodakaraensis* that lacks these residues, yet is potently inhibited by tryptophan, was recently purified from *E. coli* and characterized (Tang et al. 2001).

In this study, we present the expression, purification, and characterization of anthranilate synthase from *Archaeoglobus fulgidus* (Stetter et al. 1987), a sulfate-reducing hyperthermophile (optimum growth temperature 83°C) that has been found in submarine hydrothermal vent systems (Huber et al. 1990) and marine deep oil wells (Stetter et al. 1993). The recombinant enzyme was purified from a heterologous *E. coli* host as an active $\alpha_2\beta_2$ heterotetrameric complex and characterized in terms of its kinetic and molecular properties. Our results show that the enzyme is highly thermostable in the presence of the extrinsic factors glycerol and potassium chloride and is potently, though non-cooperatively, regulated by the feedback inhibitor tryptophan.

Materials and methods

Materials

The genomic DNA used to PCR-clone the *trpE* and *trpG* genes in tandem was isolated from *A. fulgidus* biomass provided as a gift by Harold Monbouquette and Imke Schroeder (respectively Department of Chemical Engineering, Department of Microbiology and Molecular Genetics, University of California at Los Angeles). Chorismic acid of high purity used in activity assays was either obtained from Sigma Chemical Co. or produced biosynthetically in-house.

PCR amplification and subcloning

The genome of *A. fulgidus* has been completely sequenced; and anthranilate synthase *trpE* and *trpG* genes have been identified as part of a CDEGFBA *trp* operon within the genome (Klenck et al. 1997). Within the operon, all of the genes overlap by at least one nucleotide; and the *trpE* and *trpG* genes overlap by one nucleotide such that the "A" of the CUA codon (a leucine) at the 3′ end of *trpE* overlaps with the "A" of the AUG initiation codon of *trpG*. Thus, the gene organization of the *trp* operon of *A. fulgidus* is similar to that of *Pyrococcus kodakaraensis*, which has the same order of genes (CDEGFBA) that all overlap and which is expressed as a single 6.6-kb mRNA transcript (Tang et al. 1999).

The *trpE* and *trpG* genes were PCR-amplified in tandem from *A. fulgidus* genomic DNA, using Pfu polymerase and a pair of primers designed to introduce *Nde*I and *Hin*dIII sites at the 5′ and 3′ termini, respectively. The resulting 1.8-kb PCR product was subcloned into pHisTrc99A, an expression vector produced by modifying commercially available pTrc99A (Pharmacia) such that it contained an upstream 6×His tag sequence with a thrombin cleavage site. The recombinant expression plasmid containing the *trpEG* insert was designated *trpEG*/pHisTrc99A. A ligation mixture containing the recombinant plasmid DNA was transformed into competent *E. coli* TG1 cells. The integrity of the *trpEG*/pHisTrc99A plasmid was checked by restriction mapping and by completely sequencing through the *trpE* and *trpG* coding regions, which were found to contain no mutations.

Expression

The *trpEG*/pHisTrc99A plasmid DNA was electroporated into BL21-CodonPlus-RIL *E. coli* cells (Stratagene). The CodonPlus feature of the cells was found to be essential for overcoming a codon bias problem due to the fact that one out of every 12 codons in the *A. fulgidus trpE*/*trpG* coding region is a rare (in *E. coli*) arginine codon. One-liter volumes of LB containing 100 μg ml^{-1} of ampicillin and 40 μg ml^{-1} of chloramphenicol were inoculated with 5 ml of overnight cultures and then grown at 28°C until the optical density at 600 nm reached 0.6 units (about 6 h). The mid-log phase cultures were induced with 20 μM isopropyl-β-D-thiogalactopyranoside and grown at 28°C for an additional 16 h. Cell cultures were cooled on ice and pelleted

and the pellets frozen at −20°C. Frozen cell pellets were brought up in a minimal volume of lysis buffer (50 mM sodium phosphate, pH 8.0, containing 10 mM β-mercaptoethanol, 0.15 mM phenylmethylsulfonyl fluoride). The cell suspensions were combined and the cells were broken open by three passages through a French press and then centrifuged. The supernatants were poured off, pooled, and stored at 4°C for purification of soluble recombinant *A. fulgidus* anthranilate synthase. SDS-PAGE analysis of the supernatant and the corresponding pellet fractions revealed that only a small portion of the total protein expressed was soluble. The vast majority of it went into inclusion bodies. Nevertheless, since it was reasoned that it would be difficult to unfold and refold a heterotetrameric protein having a subunit (TrpE) with a complicated fold (Knöchel et al. 1999; Morollo and Eck 2001; Spraggon et al. 2001), only soluble protein was used for the purification.

Purification

Crude extract containing soluble recombinant enzyme was heated in the presence of 20 mM glutamine for 30 min at 80 ± 5°C. The heat-treated extract was then centrifuged to remove heat-denatured protein and the supernatant was loaded onto a nickel–nitrilotriacetic acid (Ni-NTA) agarose affinity column that had been equilibrated with lysis buffer at 4°C. This column was then either washed with buffer A (lysis buffer containing 100 mM NaCl) containing 10 mM imidazole and eluted with a gradient of 10–250 mM imidazole in buffer A, or washed with 20 mM imidazole in buffer A and isocratically eluted with 250 mM imidazole in buffer A. The column fractions having the highest levels of anthranilate synthase activity were pooled and dialyzed against buffer B [20 mM Tricine, pH 8.0, 1 mM dithiothreitol (DTT), 0.1 mM EDTA] to remove the imidazole. This dialyzed Ni-NTA pool was then loaded onto a DEAE Sepharose fast flow column equilibrated with buffer B at 4°C. The column was washed with buffer B and then eluted with a gradient of 0–400 mM NaCl in buffer B. The column fractions having the highest activities were pooled, dialyzed against buffer C (50 mM Tricine, pH 7.5, 1 mM DTT, 0.1 mM EDTA), and loaded onto a Sephacryl S-200 HR size exclusion column that had been equilibrated with buffer C at 4°C. The column was eluted with the same buffer and fractions having the highest activities were pooled. This most pure enzyme preparation was concentrated by dialysis in a 1:1 mixture of glycerol and buffer C; and the enzyme solution was stored at −20°C.

Enzyme activity assays

Anthranilate synthase activity (both glutamine- and ammonia-dependent) was measured by following the formation of the fluorescent product anthranilate over time at 60°C, according to the assay method of Bauerle et al. (1987). The standard 2-ml activity assay contained 250 μM chorismate, 20 mM glutamine (or 50 mM ammonium chloride for the ammonia-dependent assay), 10 mM magnesium chloride, and 0.8 μg of recombinant enzyme in 50 mM Tricine, pH 8.0. The catalytic rate constant k_{cat} (s^{-1}) was calculated using v_{max} values obtained from substrate saturation curves and a molecular mass of 136 kDa for the $\alpha_2\beta_2$ complex. Protein concentrations were measured using the Bio-Rad protein assay reagent.

Thermostability experiments

To measure the rate of thermal inactivation of recombinant *A. fulgidus* anthranilate synthase over time, a small volume of the purified enzyme in 50 mM Tricine, pH 7.8, containing 25% glycerol and, in some cases, 2 M KCl was placed in a circulating water bath at a constant temperature. Aliquots were removed at time intervals, placed on ice, and later assayed for anthranilate synthase activity at 60°C. The percent activity remaining (compared to activity prior to incubation) was plotted versus the time of incubation in the water bath. Each curve was fitted to either: (1) a single exponential decay function, or (2) a double exponential decay function, and the half-life (or half-lives) of thermal inactivation at each incubation temperature was (were) calculated. If the data fit better to the double exponential decay function, this indicated that the enzyme sample was most likely made up of two populations with different rates of thermal inactivation.

Results and discussion

Expression, purification, and molecular mass determination of recombinant anthranilate synthase

The recombinant enzyme was expressed in *E. coli* and purified in a four-step procedure that involved: (1) heat treatment at 80 ± 5°C, (2) metal affinity chromatography, (3) anion-exchange chromatography, and (4) gel filtration chromatography. Following this procedure, the yield of soluble protein was approximately 0.1 mg l^{-1} of cell culture.

This yield is similar to that obtained for *Sol. solfataricus* anthranilate synthase purified by Knöchel et al. (1999), who also reported 0.1 mg l^{-1} of culture. The reasons for the low yield in our case are unclear, but could be due to at least two factors. First, the presence of a non-consensus GAGG Shine–Dalgarno sequence (versus the AGGAGG consensus of *E. coli*) at the end of *trpE*, together with the rather long distance (11 bp) separating this sequence from the *trpG* AUG initiation codon, might have served to lower the efficiency of expression of the *trpG* transcript. (Note that *trpE* has a strong ribosome binding sequence from the vector.)

Second, it is known that mRNA transcripts from hyperthermophiles can fold into secondary structures at *E. coli* growth temperatures that impede ribosome access to its binding site. If such structures existed near the *trpG* AUG codon, TrpG expression would have been impaired. These two factors most likely served to lower the expression of TrpG, which was observed to be present at a considerably lower level than TrpE on SDS-PAGE gels before purification. It is plausible that, if the folding of the TrpE subunit required the presence of TrpG, the lack of a comparable amount of TrpG may have caused the majority of TrpE to remain unfolded and to accumulate in inclusion bodies. Finally, once soluble recombinant anthranilate synthase protein was subjected to purification (especially size exclusion chromatography, which purifies the intact complex), the stoichiometry between the TrpE and TrpG subunits, as judged by the relative intensities of Coomassie-stained bands on a SDS-PAGE gel, was approximately 1:1, as expected.

Figure 1 shows a SDS-PAGE analysis of the purified enzyme preparation, which is approximately 85% pure. Protein bands for 6×His-tagged TrpE and for TrpG are visible at 50 kDa and 18 kDa, respectively. The purified recombinant enzyme was stored at −20°C in a 1:1 mixture of glycerol and buffer C (50 mM Tricine, pH 7.5, 1 mM DTT, 0.1 mM EDTA). Additional protein bands appeared upon storage. After an initial 10–15% drop in activity, little or no loss of enzyme activity was observed over a period of several weeks when the enzyme was stored in this manner.

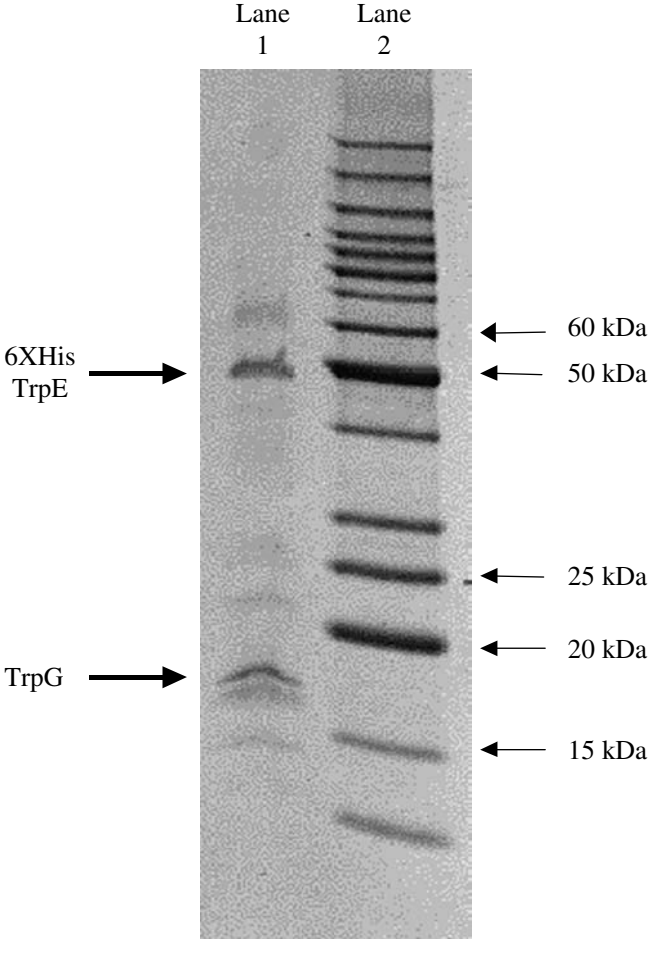

Fig. 1 SDS-PAGE analysis of purified recombinant *A. fulgidus* anthranilate synthase. *Lane 1* contains 1.8 μg of purified enzyme. *Lane 2* contains molecular mass markers (BioRad Benchmark). Sizes of selected markers are indicated on the *right*. The positions of the 6×His-TrpE and TrpG subunits are indicated on the *left*

Purified recombinant *A. fulgidus* anthranilate synthase was analyzed by gel filtration chromatography in order to determine the size of the active complex. An average molecular mass of 150 ± 15 kDa was obtained from two trials. Western blotting analysis of column fractions using anti-6×His or anti-*S. typhimurium* TrpE antibody showed that all of the detectable 6×His-TrpE was within the activity peak at this size position. This value of 150 kDa agrees well with the value of 136 kDa calculated by assuming that *A. fulgidus* is an $\alpha_2\beta_2$ heterotetramer composed of two 50-kDa 6×His-TrpE and two 18-kDa TrpG subunits.

Thermostability

The results of the thermal inactivation studies are presented in Table 1. In the presence of glycerol alone (25%), without added salt, the thermal inactivation of *A. fulgidus* anthranilate synthase followed a double exponential decay with incubation time at temperatures of 75°C and 80°C. Thus, under these conditions, the recombinant *A. fulgidus* anthranilate synthase enzyme preparation comprises two populations, one of which is 5- to 7-fold less stable than the other. At 85°C, thermal inactivation was rapid and according to a single exponential decay function, with a half-life of approximately 1 min (0.9 ± 0.2 min).

Since potassium chloride is known to have a stabilizing effect on some oligomeric proteins from hyperthermophiles (Ogasaraha et al. 1998), the effect of potassium chloride concentration on the thermostability of the recombinant anthranilate synthase was examined.

Table 1 Half-lives of thermal inactivation in either the absence or presence of 2 M potassium chloride at various temperatures. All assays contained 25% glycerol. Half-lives were determined by fitting the thermal inactivation data (activity vs time at the various temperatures) to either a single exponential decay (one half-life) or a double exponential decay (two half-lives) function, as described in the Materials and methods section. *ND* not determined

Incubation temperature (°C)	Half-life of thermal inactivation (min)	
	Without KCl	With KCl
75	4.6 ± 1.2, 35 ± 9	Activity constant for over 4 h after initial drop in activity to 83%
80	1.2 ± 0.3, 6.4 ± 0.6	200 ± 18
85	0.9 ± 0.2	50 ± 11
90	ND	12 ± 1
95	ND	4.1 ± 0.1

Thermal inactivation experiments were performed in which the enzyme was incubated at 85°C in solutions containing different concentrations of potassium chloride (0, 0.5, 1.0, 2.0 M) in Tricine buffer, pH 7.8, with 25% glycerol. The results demonstrated that the highest level of stabilization was achieved with the highest potassium chloride concentration tested (2.0 M). In order to look more closely at this thermostabilizing effect, the enzyme was incubated in a solution containing 2.0 M potassium chloride (and 25% glycerol) at different temperatures (75, 80, 85, 90, 95°C). The thermal inactivation profiles were all single exponential, indicating that a single population of anthranilate synthase enzyme molecules is active under these high salt conditions. The half-lives, displayed in Table 1, indicate that potassium chloride in the presence of glycerol significantly stabilizes the recombinant enzyme. Most dramatically, at 85°C, the half-life is almost 1 h (50 min), compared with 1 min (0.9 min) in the absence of potassium chloride. This represents a 56-fold increase in stability.

Glycerol was essential for the stabilizing effect of potassium chloride, since the enzyme was as unstable in the presence of only potassium chloride (no glycerol) as it was in the presence of only glycerol (no potassium chloride). This result, showing an important role for glycerol in thermostabilization, is reminiscent of results obtained by Sun et al. (1999), who found that glycerol stabilized recombinant *P. furiosus* glutamate dehydrogenase (a homohexamer) 14-fold at 105°C. In this case, the glycerol-induced stabilization apparently mimicked an even more dramatic pressure-induced stabilization (28-fold at 105°C). Both glycerol- and pressure-induced stabilization were proposed to follow the same mechanism, i.e., compressing and/or rigidifying the protein structure. A similar mechanism may be in effect here. One difference between the results for glutamate dehydrogenase and the results obtained for anthranilate synthase in this study, however, is that both salt and glycerol are apparently required for enhancing the thermostability of anthranilate synthase.

The thermostabilization of *A. fulgidus* anthranilate synthase by glycerol and salt is also consistent with the results of Lamosa et al. (2000), who found that diglycerol phosphate, a solute that accumulates in *A. fulgidus* cells upon salt stress, significantly stabilizes proteins such as lactate dehydrogenase, alcohol dehydrogenase, and rubredoxins. For some of the proteins studied, a 2:1 molar combination of glycerol and phosphate gave comparable stabilization. Thus, it is conceivable that the combination of charge and hydrogen bonding ability in the potassium chloride–glycerol mixture used here mimics the characteristics of compatible solutes, such as diglycerol phosphate and others (Martins et al. 1997) that are present at high levels in marine hyperthermophiles.

Removal of the 20-amino acid N-terminal histidine tag of 6×His-TrpE by thrombin cleavage did not alter the thermal stability of the recombinant enzyme. Values for the specific activity and the half-life of thermal inactivation at 75°C for the enzyme treated with and isolated away from thrombin were identical to values for an untreated control (data not shown). The efficiency of the thrombin cleavage reaction was checked by performing Western blot analysis of cleavage reaction products, using an anti-His tag antibody, and comparing the results with a negative control lacking thrombin. From this, the cleavage reaction was judged to be 90% efficient.

Catalytic properties

Steady-state kinetic constants for the recombinant anthranilate synthase were determined in the presence of glycerol (25%) and potassium chloride (2 M), since these conditions conferred maximum thermostability. These constants were also determined in the absence of glycerol and potassium chloride for comparison. The enzyme was found to obey Michaelis–Menten kinetics under both sets of conditions, giving hyperbolic saturation curves with respect to all substrates. The catalytic constants obtained included: Michaelis constants with respect to substrates, the catalytic rate constant, and the activation energy. In addition, pH optima were determined. These data are presented in Table 2 (K_m values, k_{cat} value, pH optima) and Fig. 2 (activation energy).

The values of K_m chr, K_m gln, K_m NH_4^+, k_{cat}, and the pH optima obtained for *A. fulgidus* anthranilate synthase can be compared with values obtained for anthranilate synthases from other microorganisms, namely *Sol. solfataricus* (Knöchel et al. 1999; Tutino et al. 1997), *Sal. typhimurium* (partial complex; Tamir and Srinivasan 1969), and *Ser. marcescens* (Zalkin and Hwang 1971; Robb et al. 1971). The value for K_m chr

Table 2 Catalytic properties of recombinant anthranilate synthase from *A. fulgidus* determined in either the absence or the presence of 2 M KCl and 25% glycerol. All assays were performed at 60°C, as described in the Materials and methods section. K_m and V_{max} values were determined by non-linear regression analysis of the kinetic data, using the Michaelis–Menten equation. The K_{cat} average value was obtained from saturation curves with respect to the three substrates: chorismate (*chr*), glutamine (*gln*), and ammonia

Catalytic property	Value (without KCl and glycerol)	Value (with KCl and glycerol)
K_m chr (μM)	12.3 ± 0.1	84 ± 4
K_m gln (mM)	6.2 ± 0.2	7.0 ± 0.3
K_m NH_4^+ (mM)	5.7 ± 0.3	7.3 ± 0.4
k_{cat} average at 60°C (s^{-1})	0.25 ± 0.03	0.25 ± 0.04
pH optimum of glutamine-dependent reaction	8.8 ± 0.1	8.1 ± 0.1
pH optimum of ammonia-dependent reaction	8.7 ± 0.1	8.0 ± 0.1
Tryptophan inhibition constant, K_I (μM)	0.28 ± 0.12	0.31 ± 0.01

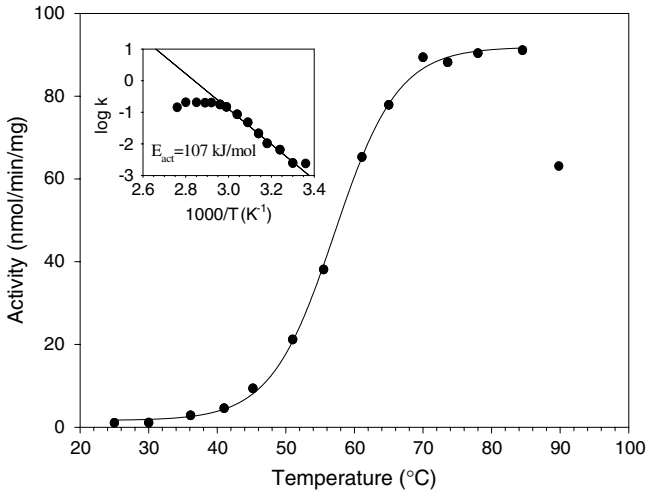

Fig. 2 Dependence of activity (nmol min^{-1} mg^{-1}) on temperature for recombinant *A. fulgidus* anthranilate synthase in the presence of glycerol (25%) and potassium chloride (2 M). *Insert* Secondary plot of log rate constant (k) versus 1/temperature (T), using the same data. The temperature range in the secondary plot is 30–65°C. The activation energy (E_{act}) was obtained from the slope of the secondary plot. Activity assays were performed at the temperature indicated on the x-axis using 0.8 μg enzyme, 250 μM chorismate, 20 mM glutamine, and 10 mM magnesium chloride, as described in the Materials and methods section

(84 μM) is considerably higher (5- to 90-fold) than those obtained for these other anthranilate synthases. The deviation of the value of K_m chr from those for other anthranilate synthases, which range from 0.9 μM to 18 μM, is due to the presence of potassium chloride, since the value in the absence of potassium chloride is 12.3 ± 0.1 μM, which is within this range. This effect of potassium chloride applies only to the utilization of chorismate, not to the utilization of other substrates or the catalytic process, since the values for the other kinetic constants determined for the *A. fulgidus* enzyme in the absence versus the presence of potassium chloride are similar (see Table 2). It is not known precisely how potassium chloride (in combination with glycerol) causes the increase in K_m chr. However, given that potassium chloride also has a profound effect on thermostability, the effect here may be through structural changes at the level of the entire tetrameric complex.

The value of K_m NH_4^+ in the presence of potassium chloride and glycerol (7.3 mM) is similar to those (9.3, 5.0, 5.9 mM, respectively) for the other anthranilate synthases. However, the value of K_m gln (7.0 mM) is about 10-fold higher than those for *Sal. typhimurium* (partial complex) and *Ser. marcescens* anthranilate synthases and is about 300-fold higher than that for *Sol. solfataricus* anthranilate synthase. This shows that glutamine is a poorer substrate for *A. fulgidus* anthranilate synthase than for the enzyme from these other microorganisms. In contrast, the similarity between the values of K_m gln and K_m NH_4^+ for *A. fulgidus* anthranilate synthase indicates that ammonia is as good a substrate as glutamine. Thus, the *A. fulgidus* enzyme is different from the other anthranilate synthases in this respect: for these other enzymes, ammonia is a much poorer substrate. The precise significance of this is not clear, but it could have implications about the structure of the active sites, or the way the sites are linked, compared with those of other anthranilate synthases.

The catalytic rate constant k_{cat} for *A. fulgidus* anthranilate synthase, 0.25 s^{-1} at 60°C, is similar to that of *Sol. solfataricus* anthranilate synthase at 60°C (0.14 s^{-1}; Knöchel et al. 1999), but is 20- to 40-fold lower than the rate constants for the mesophilic enzymes at 25°C or 35°C.

The pH optimum in the absence of glycerol and potassium chloride is approximately 8.7 for both glutamine-dependent and ammonia-dependent reactions; and, in the presence of these solutes, it is approximately 8.0 for both reactions. These results, showing similar pH optima for glutamine- and ammonia-dependent reactions, are consistent with those showing similar values of K_m gln and K_m NH_4^+; and they reflect the theme that glutamine and ammonia are utilized similarly by the enzyme. In contrast, anthranilate synthases from *Sal. typhimurium* (Zalkin and Hwang 1971) and *Ser. marcescens* (Robb et al. 1971) have pH optima around 7.5 for the glutamine-dependent reaction and 8.6 for the ammonia-dependent reaction, indicating a clear difference in the way these two substrates are utilized.

The temperature-dependence of the *A. fulgidus* anthranilate synthase-catalyzed reaction was studied. The results are presented in Fig. 2. Typical of enzymes from hyperthermophiles (Adams 1993), activity is negligible at 25°C, but increases with temperature until a maximum is reached at a $T_{optimum}$ of 85°C. The Arrhenius plot of the same data (Fig. 2, insert) is non-linear with a discontinuity at 65°C, which presumably indicates a temperature-dependent conformational transition at this temperature. The activation energy obtained from the plot between 25°C and 70°C is 107 ± 11 kJ mol^{-1}.

Tryptophan inhibition

Feedback inhibition of recombinant *A. fulgidus* anthranilate synthase by tryptophan with respect to chorismate in the absence or presence of glycerol and potassium chloride was investigated. Figure 3 presents double-reciprocal plots of 1/activity vs 1/[chorismate] in the presence of increasing amounts of tryptophan (Trp), from 0 μM to 10 μM, for reactions carried out in the absence of potassium chloride and glycerol. The pattern of intersecting lines clearly shows that the inhibition was competitive with respect to chorismate. In the presence of glycerol (25%) and potassium chloride (2 M), the enzyme gave similar results: inhibition was primarily competitive, with the lines of the plot converging just to the left of the y-axis. Further, similar inhibition constants were obtained from non-linear regression analysis of the two sets of data fit to the equation for competitive inhibition (Table 2): in the absence of glycerol and

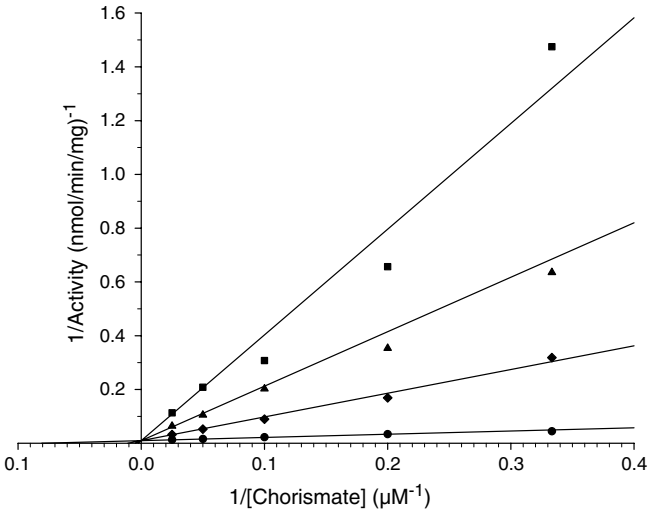

Fig. 3 Lineweaver–Burk plots showing tryptophan inhibition of recombinant *A. fulgidus* anthranilate synthase with respect to chorismate in the absence of glycerol and potassium chloride. Activity assays were performed at 60°C. Chorismate concentration was varied in the presence of the following fixed concentrations of tryptophan: none (*circles*), 2 μM (*diamonds*), 5 μM (*triangles*), and 10 μM (*squares*). All other reaction conditions were as described in the Materials and methods section

potassium chloride, K_I Trp was 0.31 ± 0.01 μM, and in the presence of these stabilizing agents, K_I Trp was 0.28 ± 0.12 μM. These values compare with 4.4 μM for *Sol. solfataricus* anthranilate synthase (Tutino et al. 1997) and 1.3 μM for the *Sal. typhimurium* enzyme (Caligiuri and Bauerle 1991a). The lack of any apparent upward curvature in the lines signifies an absence of cooperative interactions between chorismate sites within the $TrpE_2TrpG_2$ tetramer. In this respect, these results are similar to those for *Sol. solfataricus* anthranilate synthase, which likewise showed no apparent cooperativity (Tutino et al. 1997). But, they differ from those for anthranilate synthases from *Sal. typhimurium* and *Ser. marcescens*, for example, which are cooperatively regulated by tryptophan. Thus, although tryptophan is a potent competitive inhibitor of *A. fulgidus* anthranilate synthase, the inhibition does not appear to be cooperative.

Conclusion

In this paper, it is shown that extrinsic factors potassium chloride and glycerol dramatically increased the thermostability of recombinant heterotetrameric anthranilate synthase from the hyperthermophile *A. fulgidus*. The recombinant enzyme was potently, though non-cooperatively, inhibited by tryptophan. Our work highlights the importance of extrinsic thermostabilizing factors such as polyols and salts, or agents that combine the characteristics of both, such as diglycerol phosphate, for the attainment and maintenance of the proper oligomeric structures of some complex proteins from marine hyperthermophiles (Lamosa et al. 2000; Takai et al. 1997). Extrinsic factors that promote the formation of properly structured and assembled protein complexes at high temperatures may be, in some cases, as essential to the stability and function of such complex proteins as the intrinsic properties of the folded polypeptide chains themselves. As these extrinsic factors are identified and investigated, increasing numbers of complex thermostable enzymes from hyperthermophiles, such as the anthranilate synthase from *A. fulgidus* studied here, may find application in industrial biocatalysis.

Acknowledgements We wish to thank Harold Monbouquette and Imke Schroeder at the University of California at Los Angeles for giving us *A. fulgidus* biomass, Marcia Holden at National Institutes of Standards and Technology (NIST) for isolating genomic DNA from the biomass, Ron Bauerle at the University of Virginia for his gift of *Sal. typhimurium* anti-TrpE antibody and for insights into expression, and Martin Mayhew and Nick Moran at NIST for preparing the in-house chorismic acid. This work was supported in part by grant 2G12RR003048 to W.M.B. from the RCMI program of the National Center for Research Resources, U.S. National Institutes of Health.

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