A Novel Tryptophan Synthase β-Subunit from the Hyperthermophile *Thermotoga maritima*

**QUATERNARY STRUCTURE, STEADY-STATE KINETICS, AND PUTATIVE PHYSIOLOGICAL ROLE**

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Tryptophan synthase catalyzes the last two steps in the biosynthesis of the amino acid tryptophan. The enzyme is an αβα complex in mesophilic microorganisms. The α-subunit (TrpA) catalyzes the cleavage of indoleglycerol phosphate to glyceraldehyde 3-phosphate and indole, which is channeled to the active site of the associated β-subunit (TrpB1), where it reacts with serine to yield tryptophan. The TrpA and TrpB1 proteins are encoded by the adjacent *trpA* and *trpB1* genes in the *trp* operon. The genomes of many hyperthermophilic microorganisms, however, contain an additional *trpB2* gene located outside of the *trp* operon. To reveal the properties and potential physiological role of TrpB2, the *trpA*, *trpB1*, and *trpB2* genes of *Thermotoga maritima* were expressed heterologously in *Escherichia coli*, and the resulting proteins were purified and characterized. TrpA and TrpB1 form the familiar αβα complex, in which the two different subunits strongly activate each other. In contrast, TrpB2 forms a β2-homodimer that has a high catalytic efficiency *k*<sub>cat</sub>/*K*<sub>indolene</sub> because of a very low *K*<sub>indolene</sub> but does not bind to TrpA. These results suggest that TrpB2 acts as an indole rescue protein, which prevents the escape of this costly hydrophobic metabolite from the cell at the high growth temperatures of hyperthermophiles.

Hyperthermophilic microorganisms grow optimally close to the boiling point of water (1). It is interesting to identify those molecular adaptations that allow proper function of metabolism under these extreme conditions (2). In particular, enzymes from hyperthermophiles must be extremely thermostable, and labile metabolites must be protected from spontaneous degradation (3–5).

The pathway of tryptophan biosynthesis from chorismate comprises seven catalytic functions (6). In most organisms, the *trp* genes are organized in operons, which guarantees their coordinated expression in response to the amount of tryptophan available in the growth medium (7). The order of the *trp* operon from the hyperthermophile *Thermotoga maritima* *trpE*(*G)*/*CFBA* is similar to the organization of the *trp* operon from *Escherichia coli* (9). The last four steps of tryptophan biosynthesis are catalyzed by phosphoribosyl anthranilate isomerase (TrpF), indoleglycerol phosphate synthase (TrpC), and the α- and β-subunits of tryptophan synthase (TrpA and TrpB1, respectively). It was shown that TrpF and TrpC from *T. maritima* are far more thermostable than their homologs from mesophiles (10, 11), probably because of an increased association state in the case of TrpF (12, 13) and an increased number of salt bridges in the case of TrpC (11, 14). Moreover, both TrpF and TrpC from *T. maritima* are catalytically more active at 80 °C than the orthologous enzymes from *E. coli* at 37 °C, thus outrunning the unproductive hydrolysis of their thermolabile substrates under physiological conditions (10, 11, 15).

Less is known about the specific structural and functional adaptations of the tryptophan synthase from *T. maritima*, which catalyzes the conversion of indoleglycerol phosphate (IGP) and serine to tryptophan (16). The tryptophan synthases characterized so far consist of two TrpA (α) and two TrpB1 (β) structural entities, which are organized either as four monofunctional subunits or as two bifunctional αβ-subunits (17). The x-ray structure at 2.8 Å resolution of the tryptophan synthase from *Salmonella typhimurium* revealed an αβα quaternary structure (18). The structure of isolated TrpA from *Pyrococcus furiosus* confirmed that this enzyme has a β-barrel fold (19), which is the most frequently encountered topology among single domain proteins and is also adopted by TrpF and TrpC (20, 21). TrpB1 consists of two domains, which both comprise a central open β-sheet that is surrounded by α-helices (18). TrpA catalyzes the aldolytic cleavage of IGP to glyceraldehyde 3-phosphate (GA3P) and indole, which condenses with serine at the active site of TrpB1 to yield tryptophan (Fig. 1). The hydrophobic intermediate indole passes directly from the α-site to the β-site via a long tunnel, which prevents its loss from the cell by diffusion across the cytoplasmic membrane (22, 23). There is pronounced allosteric communication between the TrpA and TrpB1 subunits from *E. coli*, which is reflected in a mutual activation of their catalytic activities which keeps the two reactions in phase and prevents accumulation of indole (24). It appears that the αβα complex is in an equilibrium between a low activity “open” and a high activity “closed” state, which is shifted by allosteric ligands and monovalent cations (25). The basis of the corresponding conformational transitions has been characterized by x-ray structure analysis of a number of enzyme-ligand complexes (26–30).

Recently, the genome sequencing of *T. maritima* (31) and of other hyperthermophiles has identified a *trpB2* gene outside of the *trp* operon. To reveal the roles in tryptophan biosynthesis of the two different TrpB variants, tmTrpA, tmTrpB1, and tmTrpB2, the authors studied the properties and potential physiological role of the αββα complex.

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tmTrpB2 from *T. maritima* were expressed heterologously in *E. coli*, and the corresponding protein products were purified and characterized by hydrodynamic measurements and steady-state enzyme kinetics. The results show that tmTrpB1 associates with tmTrpA to an αβα complex, in which the two different subunits strongly activate each other. tmTrpB2, which does not bind to tmTrpA but is catalytically highly active, has an extremely low $K_m$ value for indole. It appears that tmTrpB1 has the same role in tryptophan biosynthesis as the known TrpB1 enzymes from mesophiles, whereas tmTrpB2 acts as a salvage protein that prevents the loss of indole at the physiological growth temperatures of hyperthermophiles.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulation and Sequence Analysis**—Preparation of DNA, amplification, extraction, digestion with restriction endonucleases, ligation, and sequencing were performed as described (32).

Subcloning of tmtrpA, tmtrpB1, and tmtrpB2 Genes from *T. maritima*—The genes tmtrpA and tmtrpB1 were amplified by PCR using the plasmid pDMstrpAB (16) as template. For amplification of tmtrpA, the oligonucleotides 5'-GGTTCATATGAAAGGCTTTAATGCATACTC-3' with a NdeI site (in boldface type) and 5'-CGATGAATTCCTGTTTTCGAGGGTTTCTT-3' with an EcoRI site (in boldface type) were used as 5' and 3'-primers, respectively. The tmtrpB1 gene was amplified with the primers 5'-GGATCATATGAAAAAGGTACCCGACTCTTTA-3' and 5'-CCGTGAATTCCTCATCTTATTCCCTCCTGGACGTA-3', again introducing NdeI and EcoRI sites (in boldface type). The two newly introduced restriction sites, the amplified DNA fragments were cloned into different pET vectors (Stratagene), yielding the plasmids pET21a-tmtrpA and pET24a-tmtrpB1. The gene tmtrpB2 was amplified by PCR using genomic DNA of *T. maritima* as the template. The primers 5'-ACCGCATATGAAAGGTTTGTGAA-3' and 5'-CAGGAATTCACACGTTAGGCCTTC-3' were used, introducing NdeI and HindIII sites, respectively (in boldface type). The amplified DNA fragment was cloned into the vector pET21a to yield the plasmid pET21a-tmtrpB2. All inserts were sequenced entirely to exclude inadvertent PCR mutations.

Production of tmTrpA, tmTrpB1, and tmTrpB2, the (tmTrpA-tmtrpB1) Complex, and of Glyceraldehyde-3-phosphate Dehydrogenase from *T. maritima* (tmGAPDH)—Heterologous expression of tmTrpA was conducted in *E. coli* BL21(DE3) (33) cells containing the plasmid pET21a-tmtrpA. For reasons unknown so far, tmtrpA (and tmtrpB1 and tmtrpB2) are being expressed in the absence of isopropyl-1-thio-β-D-galactopyranoside. Therefore, the cells were grown overnight at 37 °C in 1 liter of LB medium supplemented with 150 μg/ml ampicillin but without the addition of isopropyl-1-thio-β-D-galactopyranoside. The cell pellet resulting from centrifugation was resuspended in 10 ml potassium phosphate buffer at pH 7.5 and lysed by sonication (Branson Sonifier W-250, 3 × 3 min, 50% pulse, 0 °C). According to SDS-PAGE, 90% of tmTrpA was found in the soluble fraction of the cell extract. 100 units of benzonase (Merck) was added to this fraction, which was then incubated for 1 h at 37 °C to degrade nucleic acids and subsequently for 20 min at 75 °C to denature the benzonase. The resulting suspension was centrifuged (Sorval SS34, 13,000 rpm, 30 min, 4 °C), and the pellet, which contained heat-labile host proteins, was discarded. The supernatant was loaded on an anion exchange column (Mono Q, 2 × 11 cm, Amersham Biosciences, Inc.) that was equilibrated with 10 mM potassium phosphate buffer, pH 7.5, at room temperature. The column was washed with 4 column volumes of equilibration buffer, and bound proteins were eluted with a linear gradient of 0–750 mM potassium chloride at pH 7.5. tmTrpA eluted at 150–200 mM potassium chloride, as judged from SDS-PAGE and conductivity measurements. Sufficiently pure fractions were pooled, concentrated using Centricon-10 devices (Millipore), and dialyzed against 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM potassium chloride. The protein was shock frozen in liquid nitrogen at a concentration of 10 mg/ml. The purification yielded 50 mg of tmTrpA out of 1 liter of cell culture with a purity of about 99% as judged by SDS-PAGE. For expression of tmtrpB1 and tmtrpB2, *E. coli* BL21(DE3) cells containing the plasmids pET24a-tmtrpB1 and pET21a-tmtrpB2 were used. Cells were grown as described for tmtrpA, with the exceptions that kanamycin instead of ampicillin was added for maintenance of pET24a-tmtrpB1 and 20 °C instead of 37 °C was used, which increased the fraction of soluble protein to about 40% for tmTrpB1 and about 10% for tmTrpB2 (33). Harvesting of cells, cell lysis, incubation with benzoyl-arginine ethyl ester, heat precipitation of host proteins, and anion exchange chromatography were performed as described for tmTrpA, with the exception that the buffer solutions were supplemented with 40 μM pyridoxal 5'-phosphate (PLP). Both tmTrpB proteins eluted from the Mono Q column at about 150–200 mM potassium chloride. Fractions containing either tmTrpB protein were pooled, concentrated using Centricon-10 devices, and loaded on a gel filtration column (Superdex 75, HiLoad 26/60, Amersham Biosciences, Inc.) equilibrated with 50 mM potassium phosphate at pH 7.5, containing 300 mM potassium chloride and 40 μM PLP. The tmTrpB proteins, which eluted with a purity above 95% as judged from SDS-PAGE, were shock frozen in liquid nitrogen at concentrations of 4 mg/ml (tmTrpB1) and 1.5 mg/ml (tmTrpB2). From 1 liter of cell culture, 16 mg of tmTrpB1 and 6 mg of tmTrpB2 were obtained.

The tmgapdh gene cloned into the plasmid pKM1 was expressed using *E. coli* BL21(DE3) cells (34). The cells were grown at 37 °C in 1 liter of LB medium supplemented with 150 μg/ml ampicillin, inducted with 1 mM isopropyl-1-thio-β-D-galactopyranoside at $A_{600}$ = 0.6, and incubated overnight. Harvesting of cells, cell lysis, and incubation with benzoyl-arginine ethyl ester were performed in a way similar to that described for tmTrpA, but a 2 mM EPPS buffer at pH 7.5 was used instead of 10 mM potassium phosphate. After heat precipitation of host proteins at 75 °C for 30 min, tmGAPDH was pure to 90%. The protein was dialyzed against 10 mM EPPS buffer at pH 7.5, containing 10 mM potassium chloride, concentrated to 5.2 mg/ml using Centricon-10 devices, and shock frozen in liquid nitrogen. From 1 liter of cell culture, 14 mg of tmGAPDH was obtained.
Fig. 2. TrpB proteins can be divided into two sequence families. The phylogenetic tree shown is based on a multiple amino acid sequence alignment performed with PILEUP (GCG Wisconsin package). The TrpB1 family contains representatives from mesophiles (regular type) and from hyperthermophilic microorganism (boldface type), the TrpB2 family contains representatives only from hyperthermophilic microorganism. TrpB1 and TrpB2 from *T. maritima* are underlined. Symbols: gixxxx, accession number in the NCBI Protein Data Base; A, archaeon; B, bacterium. The full species names can be obtained from the Protein Data Base.

**Analytical Methods**—Purification of proteins was followed by electrophoresis on 12.5% SDS-polyacrylamide gels using the system of Laemmli (35) and staining with Coomassie Blue. The concentration of tmTrpA was determined using the molar extinction coefficient ε_{280} = 18.9 m\textsuperscript{-1} cm\textsuperscript{-1}, which was calculated from the amino acid sequence (36). The concentrations of tmTrpB1 and tmTrpB2 were determined according to Bradford (37) because the strong absorption at 280 nm of the bound cofactor PLP impedes a reliable calculation of ε_{280} from the amino acid sequences.

Analytical gel filtration was performed at a flow rate of 0.5 ml/min on a Superdex 75 column (1 × 30 cm) that was equilibrated with 50 mm potassium phosphate buffer at pH 7.5 at 25 °C containing 300 mm potassium chloride. Apparent molecular masses were calculated from the corresponding elution volumes, using a calibration curve that was obtained with standard proteins. Sedimentation velocity and sedimentation equilibrium runs were performed at 20 °C in a Beckman analytical ultracentrifuge (model Optima XLA), monitoring the absorbance at 277 nm. The velocity runs were performed at 54,000 (tmTrpA) or 52,000 rpm (other proteins) and the equilibrium runs at 22,000 rpm (tmTrpA) or 8,000 rpm (other proteins). The proteins were dissolved in 100 mm potassium phosphate at pH 7.5, containing 180 mm potassium chloride and 40 μL PLP. For analysis of the equilibrium runs, a floating baseline computer program that adjusts the baseline absorbance (A) was used to obtain the best linear fit of ln(A) versus the square of the radial distance (r²). Molecular masses were calculated assuming a partial specific volume of 0.73 ml/g.

**Steady-state Enzyme Kinetics**—The cleavage of IGP to GASP and indole (A-reaction; Fig. 1) was measured under steady-state conditions between 30 and 60 °C in a coupled enzymatic assay (38). In this assay, arsenate and GASP produced by tmTrpA were converted by tmGAPDH into 1-arseno-3-phosphoglycerate upon reduction of NAD\textsuperscript{+} to NADH. The reaction is irreversible because of the spontaneous hydrolysis of 1-arseno-3-phosphoglycerate into arsenate and 3-phosphoglycerate. Initial velocities were measured using absorption spectroscopy and analyzed using Δε_{290} (NADH – NAD\textsuperscript{+}) = 6.22 m\textsuperscript{-1} cm\textsuperscript{-1}. Initial velocities were measured, and V\textsubscript{max} and K_{m} were determined with a direct linear plot (39). The conversion of indole and serine to tryptophan (B-reaction; Fig. 1) catalyzed by tmTrpB1 or tmTrpB2 was measured at 80 °C by absorption spectroscopy and analyzed using Δε_{290} (Trp – indole) = 1.89 m\textsuperscript{-1} cm\textsuperscript{-1} (40). Initial velocities were measured as a function of the concentration of either indole or serine, with the other substrate being present at saturating concentrations. In the case of tmTrpB2, entire progress curves at saturating concentrations of serine were analyzed with the integrated form of the Michaelis-Menten equation (41), which allowed determination of the upper limit of K_{m}. The conversion of IGP and serine to tryptophan (AB-reaction; Fig. 1) catalyzed by [tmTrpA·tmTrpB1] between 30 and 60 °C was followed by absorption spectroscopy and analyzed using Δε_{290} (Trp – IGP) = 0.56 m\textsuperscript{-1} cm\textsuperscript{-1} (42). Alternatively, the AB-reaction was followed in a coupled reaction, which is similar to that used to follow the A-reaction (43). Initial velocities were measured as a function of the concentration of either IGP or serine, with the other substrate being present at saturating concentrations. The K_{cat} and V_{max} values of the A- and the AB-reactions determined between 30 and 60 °C were extrapolated to 80 °C by an Arrhenius plot.

**RESULTS**

The Genomes of Many Hyperthermophiles Contain Two Different trpB Genes

The genes trpA and trpB1 of the hyperthermophilic bacterium *T. maritima* are adjacent in the trp operon (8). The sequencing of the whole genome of *T. maritima* (31) identified a gene outside of the trp operon, which has significant sequence similarity to trpB1 and was designated trpB2. It is likely that the trpB2 gene is expressed in *T. maritima* because the upstream region on the genome contains consensus sequences that are typical of bacterial promoters and ribosome binding sites (data not shown). A data base search revealed that trpB2 genes are also present in the genomes of most of the other investigated hyperthermophilic Bacteria and Archaea but are generally absent from the genomes of mesophiles. A phylogenetic tree based on amino acid sequence comparisons shows that TrpB1 and TrpB2 proteins form two separate groups (Fig. 2). Within the two groups, proteins display sequence identities of about 60%, whereas between members from different groups the identities are only about 30%. Most hyperthermophiles contain one TrpB1 and one TrpB2 protein; others, for example *Sulfolobus solfataricus*, possess two different TrpB2 variants but lack TrpB1. Fig. 3a presents the amino acid sequence alignment of the two TrpB variants from *T. maritima*, tmTrpB1 and tmTrpB2, which show an overall identity of 38%. It is evident that those amino acids, which are conserved both
in the TrpB1 and TrpB2 sequences, cluster at the putative active sites. In contrast, amino acids that are conserved in only one of the two TrpB groups are distributed along the sequences. The major differences between the two proteins are a long N-terminal extension and two shorter insertions in tmTrpB2, which are located in regions where tmTrpB1 interacts with tmTrpA, as deduced from the structure of S. typhimurium tryptophan synthase complex (Fig. 3b).

Production and Purification of tmTrpA, tmTrpB1, and tmTrpB2

The tmtrpA, tmtrpB1, and tmtrpB2 genes were cloned into different pET vectors and expressed heterologously in E. coli BL21(DE3) cells (44). tmTrpA could be produced in soluble form at 37 °C, but tmTrpB1 and tmTrpB2 had to be expressed at 20 °C to suppress in part the formation of insoluble aggregates (33). The resulting thermostable tmTrpA, tmTrpB1, and tmTrpB2 proteins were purified from the soluble fraction of the cell extract, using a heat step to remove thermolabile host proteins followed by ion exchange chromatography. The three proteins were more than 95% pure, as judged from SDS-PAGE (data not shown). The [tmTrpA-tmTrpB1]2 complex was produced by mixing tmTrpA and tmTrpB1 (see below).

Association States and Complex formation of tmTrpA, tmTrpB1, and tmTrpB2

Analytical gel filtration on a calibrated Superdex 75 HR column was used to test whether tmTrpB1 and tmTrpB2 form a complex with tmTrpA at 25 °C. The results are summarized in Fig. 4. Separately, tmTrpA, tmTrpB1, and tmTrpB2 elute as well defined peaks. When tmTrpB1 is mixed with a molar excess of tmTrpA, the tmTrpB1 peak is replaced by a new and faster elution peak, which represents a complex of tmTrpA and tmTrpB1 (Fig. 4a). In contrast, the elution profile of a mixture of tmTrpA and tmTrpB2 excludes any significant complex formation between these proteins (Fig. 4b). The elution time of separated tmTrpA corresponds to a molecular mass of 26.8 kDa, comparing well with the calculated molecular mass for the monomer (26.7 kDa). The elution times of tmTrpB1 and tmTrpB2, however, correspond to molecular masses of 49.4 and 61.7 kDa, respectively, which are between the calculated molecular masses for the respective monomers (42.9 and 46.4 kDa) and homodimers (85.8 and 92.8 kDa). Analytical ultracentrifugation was therefore performed to clarify the association states of tmTrpB1 and tmTrpB2 and to assess the stoichiometry of the complex between tmTrpA and tmTrpB1 (Table I). Sedimentation velocity runs showed that the separated proteins are homogeneous species, yielding sedimentation coefficients of 2.8 for tmTrpA and 5.4 for both tmTrpB1 and tmTrpB2. The analysis of sedimentation equilibrium runs confirms that separated tmTrpA exists mainly as an α-monomer and shows that both tmTrpB1 and tmTrpB2 are β-dimers. Runs that were performed with a mixture of tmTrpA and tmTrpB1 show that they form an αβα complex, as observed for other investigated tryptophan synthases (6, 45). In accordance with analytical gel filtration, analytical ultracentrifugation detected no complex formation between tmTrpA and tmTrpB2.

Heat Stabilities

To test their stability against irreversible inactivation by heat, tmTrpA, tmTrpB1, tmTrpB2, and the αβα complex of tmTrpA with tmTrpB1 were incubated at 85 °C. Samples with-
drawn after different time intervals were chilled on ice, and their residual catalytic activities were measured at 60 °C. For all proteins, a time-dependent monoeponential decay of the catalytic activity was observed. The measured half-lives were 125 min for tmTrpA, 320 min for both tmTrpB1 and tmTrpB2, and 40 min for the αββα complex. These results suggest that all proteins are very thermostable and that complex formation further stabilizes both tmTrpA and tmTrpB1.

Steady-state Enzyme Kinetics

The catalytic activities at 80 °C of separated tmTrpA (A-reaction), separated tmTrpB1 and tmTrpB2 (B-reaction), and of the αββα complex of tmTrpA and tmTrpB1 (A-reaction, B-reaction, and AB-reaction, Fig. 1) were determined under steady-state conditions.

A-reaction—Table II shows that the catalytic efficiency $k_{cat}/K_m^{\text{GIP}}$ of tmTrpA is increased about 270-fold by complex formation with tmTrpB1 because of an increase of $k_{cat}$ and a decrease of $K_m^{\text{GIP}}$. The strength of the activation of tmTrpA by tmTrpB1 increases with temperature from 30 to 60 °C, yielding a linear relationship in the Arrhenius plot (data not shown). As a consequence, tmTrpA is activated at 80 °C by tmTrpB1 to a similar extent as ecTrpA is activated by ecTrpB at 25 °C (Table II).

Analytical gel filtration and analytical ultracentrifugation performed at room temperature suggest that tmTrpA and tmTrpB2 do not form a complex (Fig. 4b and Table I). To test whether such a complex might be formed at higher temperatures, the catalytic activity of tmTrpA was measured at 60 °C in the presence and absence of an equimolar concentration of tmTrpB2. Because the presence of tmTrpB2 did not affect the tmTrpA activity, the formation of a functional complex between tmTrpA and tmTrpB2 can be excluded both at 25 and at 60 °C.

B-reaction—The $B$-activities of separated tmTrpB1 (β2) and of tmTrpB1 in the [tmTrpA×tmTrpB1]ν complex are compared in Table III, which shows that tmTrpB1 is activated by tmTrpA. Whereas $k_{cat}$ and $K_m^{\text{GIP}}$ are only slightly improved by complex formation, $K_m^{\text{IND}}$ is significantly decreased, and, as a result, $k_{cat}/K_m^{\text{IND}}$ is increased 65-fold. Remarkably, by complex formation with the corresponding TrpA subunits, tmTrpB1 at 80 °C and ecTrpB at 25 °C are activated to a similar extent. This result means that at the corresponding physiological temperatures, the mutual activation of the α- and the β-subunits in the αββα complex is comparably strong in the enzymes from T. maritima and E. coli. This finding supports the concept of “corresponding states,” which postulates that mesophilic and

![Panel a](image1.png) ![Panel b](image2.png)
E. coli lower in the AB-reaction compared with the A- and B-reactions, E. coli AB-reaction in the same order of magnitude.

Km of tmTrpB2 is much lower compared with complexed tmTrpB1 of the A-reaction, but about 10-fold lower than the kcat of complexed tmTrpB1 (Table IV) because both kcat and Km of the AB-reaction of the tryptophan synthase complex from T. maritima are comparably flexible, stable, and active at their respective physiological temperatures (3, 46–48).

Activity measurements with tmTrpB2 (β2) showed the absence of significant activation by tmTrpA, as expected (data not shown). The kcat/KmND of separated tmTrpB2 is similar to that of complexed tmTrpB1 (Table IV) because both kcat and KmND of tmTrpB2 are decreased by about 1 order of magnitude compared with tmTrpB1. This result suggests that tmTrpB2 binds indole much more tightly than tmTrpB1 but converts it more slowly to tryptophan. In contrast, kcat/KmSER of separated tmTrpB2 is much lower compared with complexed tmTrpB1 because kcat is decreased, and KmSER is increased by about 1 order of magnitude.

**AB-reaction**—In the AB-reaction (Fig. 1), the αββγ complex catalyzes the conversion of IGP and serine to tryptophan. Table V summarizes the steady-state enzyme kinetic constants kcat, Kcat, Kcat’/Kcat, and Kcat’/Kcat of the [tmTrpA·tmTrpB1]2 complex at 80 °C and the E. coli [ecTrpA·ecTrpB1]2 complex at 25 °C. For comparison, the values of the A- and B-reaction of the complex (see Tables II and III) are also listed. The kcat value of the AB-reaction in the E. coli complex is much higher than the kcat of the A-reaction, but about 10-fold lower than the kcat of the B-reaction. The rate of the AB-reaction of the tryptophan synthase complex from E. coli thus appears to be limited by the rate of the A-reaction. In contrast, in the T. maritima tryptophan synthase complex the kcat values of the A-, the B-, and the AB-reactions are similar, suggesting that both partial reactions influence the rate of the overall reaction. Moreover, in the T. maritima tryptophan synthase both Kcat’/Kcat and Kcat’/Kcat are much lower in the AB-reaction compared with the A- and B-reactions, respectively. In contrast, in the E. coli enzyme both Kcat’/Kcat and Kcat’/Kcat are similar in the overall and in the individual reactions. The mechanistic basis of these differences between the T. maritima and E. coli tryptophan synthases has yet to be elucidated.

**DISCUSSION**

The phylogenetic tree depicted in Fig. 2 shows that TrpB1 and TrpB2 proteins form two separate groups, which presumably evolved independently from each other after an early gene duplication event. In separated form both tmTrpB1 and tmTrpB2 are homodimers (Table I) and have similar amino acid sequences at their putative active sites containing all residues that are catalytically essential for the TrpB reaction (Fig. 3a and Ref. 17). In accordance with these observations, both tmTrpB variants catalyze the B-reaction with high efficiency at 80 °C (Table IV). Moreover, the tmTrpB1 gene is part of the trp operon of T. maritima (8), and the upstream sequence of the tmtrpB2 gene contains a putative promoter and a ribo-some binding site (31). It therefore has to be assumed that both tmTrpB1 and tmTrpB2 are produced in T. maritima and play a functional role in tryptophan biosynthesis.

The most significant difference between the two tmTrpB proteins is that tmTrpA forms a functional complex only with tmTrpB1 (Tables I–III and Fig. 4). Sequence alignment shows that tmTrpB2 contains additional amino acids compared with tmTrpB1 (Fig. 3a), which are inserted at sites where TrpA and TrpB interact with each other (Fig. 3b). It appears therefore that the binding of tmTrpA to tmTrpB2 is prevented by sterical hindrance caused by these insertions. Remarkably, maize contains two enzymes of secondary metabolism which show significant sequence similarities to TrpA and catalyze the production of indole from IGP efficiently in the absence of TrpB (49). Sequence deviations of these TrpA homologs have been identified in the intermolecular interaction domain, which might prevent their complex formation with TrpB.

Whereas tmTrpB1 is likely to receive indole from tmTrpA by intermolecular channelling (22, 23), the cellular source of indole to be used by tmTrpB2 is not evident. In E. coli, the cleavage of tryptophan as the sole carbon source to pyruvate and indole is the main source for indole (50). This reaction is catalyzed by the enzyme tryptophanase, but no gene with significant sequence similarity to known tryptophanase genes appears to be present on the genome of T. maritima (31). Alternatively, at 80 °C IGP might be spontaneously degraded with a significant rate into GA3P and indole, which could then be used by tmTrpB2 for tryptophan biosynthesis. However, at 80 °C no conversion of IGP into tryptophan could be detected in the presence of tmTrpB2 (or tmTrpB1) and serine (data not shown). It has been shown for the E. coli tryptophan synthase that less than 1% of the indole produced by ecTrP is released into the solvent at 25 °C instead of being channeled to ecTrP (51, 52). It is possible, however, that a larger fraction of indole leaks from the channel connecting tmTrP with tmTrP at the high physiological temperature of T. maritima. Consistent with this hypothesis, tmTrP2, because of its extremely low KmND (Table IV), would be ideally suited to scavenge the liberated indole and to prevent it from penetrating through the cytoplasmic membrane (Fig. 5). The putative function of tmTrP2 as an indole salvage protein is supported by the observation that trpB2 genes are only found in hyperthermophiles (Fig. 2), the high physiological temperatures of which promote passive diffusion of metabolites significantly. Some hyperthermophiles such as S. solfataricus possess two trpB2 genes but lack a trpB1 gene (Fig. 2), and it remains to be tested whether one of the two TrpB2 proteins forms an αββγ tryptophan synthase complex with TrpA. In mesophilic organisms the trpB2 gene might have been lost in the course of evolution because
of the lack of selective pressure, provided that life began in boiling water (53).

Acknowledgments—We thank Ariel Lustig for running the analytical ultracentrifuge, Steffi Hentzelt and Halina Szadkowski for the expression plasmids and [tmTrpA·tmTrpB1]2 protein, Dr. Wolfgang Liebl for a critical reading of the manuscript. We appreciate discussions with Drs. Charles Yanofsky and Birte Hocker for a critical reading of the manuscript.

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Thermotoga maritima Tryptophan Synthase