Chorismate Synthase from the Hyperthermophile \textit{Thermotoga maritima} Combines Thermostability and Increased Rigidity with Catalytic and Spectral Properties Similar to Mesophilic Counterparts*

Received for publication, January 30, 2001, and in revised form, March 6, 2001
Published, JBC Papers in Press, March 9, 2001, DOI 10.1074.jbc.M100867200

Teresa B. Fitzpatrick‡, Philipp Killer‡, Richard M. Thomas§, Ilian Jelesarov¶, Nikolaus Amrhein†, and Peter Macheroux‡

From the ‡ETH-Zürich, Institut für Pflanzenwissenschaften, Universitätstrasse 2, CH-8092 Zürich, Switzerland, the §ETH-Zürich, Institut für Polymere, Universitätstrasse 6, CH-8092 Zürich, Switzerland, and the ¶Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Chorismate synthase, the last enzyme in the shikimate pathway, catalyzes the transformation of 5-enolpyruvylshikimate 3-phosphate to chorismate, a biochemically unique reaction in that it requires reduced FMN as a cofactor. Here we report on the cloning, expression, and characterization of the protein for the first time from an extremophilic organism \textit{Thermotoga maritima} which is also one of the oldest and most slowly evolving eu-bacteria. The protein is monofunctional in that it does not have an intrinsic ability to reduce the FMN cofactor and thereby reflecting the nature of the ancestral enzyme. Circular dichroism studies indicate that the melting temperature of the \textit{T. maritima} protein is above 92 °C compared with 54 °C for the homologous \textit{Escherichia coli} protein while analytical ultracentrifugation showed that both proteins have the same quaternary structure. Interestingly, UV-visible spectral studies revealed that the dissociation constants for both oxidized FMN and 5-enolpyruvylshikimate 3-phosphate decrease 46- and 10-fold, respectively, upon heat treatment of the \textit{T. maritima} protein. The heat treatment also results in the trapping of the flavin cofactor in an apolar environment, a feature which is enhanced by the presence of the substrate 5-enolpyruvylshikimate 3-phosphate. Nevertheless, stopped-flow spectrophotometric evidence suggests that the mechanism of the \textit{T. maritima} protein is similar to that of the \textit{E. coli} protein. In essence, the study shows that \textit{T. maritima} chorismate synthase exhibits considerably higher rigidity and thermostability while it has conserved features relevant to its catalytic function.

Chorismate synthase catalyzes the final step in the shikimate pathway, which links the metabolism of carbohydrates to the biosynthesis of the three aromatic amino acids and many aromatic secondary metabolites in a series of seven enzymatic steps. The pathway is absent from mammals making it a prime target for the development of antimicrobial and herbicidal agents. Chorismate synthase itself is biochemically unique in nature in that it catalyzes a 1,4-anti-elimination of the 3-phosphate group and the 6 (pro-R)-hydrogen from 5-enolpyruvylshikimate 3-phosphate (EPSP)\textsuperscript{1} to yield chorismate (1, 2). There is no other example of this type of catalysis known in nature, thereby making it exclusive. The enzyme has an absolute requirement for reduced FMN (3, 4) and can be classified with regard to how it acquires this essential cofactor. The chorismate synthases for which the reduced flavin has to be supplied exogenously are referred to as monofunctional, \textit{e.g.} those from \textit{Escherichia coli} and plants (3, 5–9), while chorismate synthases which possess the intrinsic ability to reduce the flavin at the expense of NADPH are referred to as being bifunctional, \textit{e.g.} the \textit{Neurospora crassa} enzyme (4, 10, 11). From an evolutionary point of view, while it has been concluded from a phylogenetic analysis that all chorismate synthases are of monophyletic origin, it is not clear if the ancestral chorismate synthase was mono- or bifunctional (12). It has been suggested that the ancestral enzyme harbored the intrinsic flavin reduction activity (\textit{i.e.} was bifunctional) as it is hard to imagine how this activity could have evolved in a framework of what are known to be monofunctional enzymes, \textit{i.e.} the bacterial and plant chorismate synthases (12). In any case, as reduction of the FMN cofactor could be envisioned as a possible regulatory effector of chorismate synthase it would be intriguing to establish the history of how the enzyme has maintained reduction of the cofactor.

Recently, the genome of the hyperthermophilic bacterium \textit{Thermotoga maritima} (\(T_{\text{max}} = 90 °C, T_{\text{opt}} = 80 °C\)) has been completed and 24% of its genes have been reported to be more similar to archaean genes than to other bacterial genes (13). As this is a much higher percentage than that found in mesophilic bacteria (3–7%), it has been suggested that considerable lateral gene transfer has occurred between archaean and \textit{T. maritima} (13). In fact, \textit{T. maritima} is believed to be one of the oldest and most slowly evolving lineages in the eu-bacteria (14). Therefore, classification of \textit{T. maritima} chorismate synthase with regard to how it acquires the reduced FMN cofactor can be considered to reflect the nature of the ancestral enzyme. In addition, thermophilic proteins, as a result of residing in an extreme environment, are more stable and tend to display greater conformational rigidity than mesophilic proteins. Therefore, a comparison of the characteristics of a thermophilic chorismate synthase with that of a mesophilic counterpart may provide insights not only into the stability but also on the self-organ-

\footnote{1 The abbreviations used are: EPSP, 5-enolpyruvylshikimate 3-phosphate; IPTG, isopropyl-1-thio-β-D-galactopyranosidase; PAGE, polyacrylamide gel electrophoresis.}

* This work was supported by the Swiss National Science Foundation (to N. A. and P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\* To whom correspondence should be addressed. E-mail: peter.macheroux@ipw.biol.ethz.ch; Tel: 41-1-6327827; Fax: 41-1-6321044.
zation of this enzyme. Here we report on the cloning, expression, and purification of chorismate synthase from the thermophilic eubacterium *T. maritima*. This is the first time that this enzyme has been described from an extremophilic organism. We show that the enzyme is monofunctional with respect to its requirement for FMN indicating that the ancestral protein was in fact monofunctional rather than bifunctional as had been proposed previously (12). In addition, we describe the characteristics of the thermophilic enzyme compared with those of the mesophilic *E. coli* chorismate synthase. In particular, we address the thermal stability as well as the spectrophotometric and electrophoretic properties of both the mesophilic and thermophilic enzymes in the presence and absence of ligands. We also report on the quaternary structure of *T. maritima* chorismate synthase.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Restriction endonucleases and DNA modification enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany) or New England Biolabs (Beverly, MA). Oligonucleotide sequencing and polymerase chain reaction primers were obtained from Microsynth (Balgracht, Switzerland). All other chemicals were of the highest grade available.

**Molecular Techniques**—Basic molecular techniques were adopted from Ausuble et al. (15) or Sambrook et al. (16).

**Cloning and Expression of *T. maritima* Chorismate Synthase**—Cell paste (1 g) of the thermophilic organism *T. maritima* (strain MS88) was kindly provided by Dr. Huber (University of Regensburg, Germany). The genomic DNA was isolated using the QiAamp Tissue kit (Qiagen) according to the instructions described by the manufacturer. The DNA sequence of the *T. maritima* *aroC* gene which codes for chorismate synthase was obtained from GenBank™ (accession number AE001715.1). The *aroC* gene was amplified by the polymerase chain reaction (PerkinElmer Life Sciences) (5-primer, GGAATTCCATATG-AAAGCTTACCGGCGATGTTT; 3-primer, CCGAAGCTTTACA CGATAGGCCTCTTCCAAGAC) based on the MS88 strain sequence and cloned into the NdeI and HindIII restriction sites, respectively, of pET21a (Novagen). This vector allows expression of *T. maritima* chorismate synthase under control of the IPTG inducible T7 promoter. The expression construct was verified by sequence analysis and transformed into either *E. coli* BL21(DE3) or *E. coli* BL21-CodonPlus(RI)-RIL cells (Stratagene). For large scale expression, the single transformant was grown in 5 ml of 2 × YT medium supplemented with 100 μg/ml ampicillin and this was used to inoculate a 50-ml culture. After 1 h at 37 °C, expression was induced by addition of IPTG for a final concentration of 0.1 mM. Cells were grown to allow for another 5 h at 37 °C and then harvested by centrifugation and subsequently stored at −80 °C.

**Expression and Purification of Recombinant *T. maritima* Chorismate Synthase**—Either a crude extract (as obtained from IPTG induced expression of *E. coli* BL21 CodonPlus(DE3)-RIL cells harboring the constructed pET-T. maritima *aroC* plasmid) or the purified protein was subjected to 10% SDS-PAGE. The band of interest was excised and subjected to automated Edman degradation in an Applied Biosystems 477A sequencer.

**Immunochemical Methods**—For Western blot analysis, the samples were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with TBS (10 mM Tris-HCL, pH 7.5, 0.9% sodium chloride) supplemented with 5% dry skimmed milk and 0.05% Tween 20. The blot was incubated for 90 min with an affinity purified antibody raised against chorismate synthase from the higher plant *Corydalis sempervirens* at a suitable dilution in the blocking buffer. The membrane was washed five times for 10 min each in TBST (TBS containing 0.05% Tween 20). It was then incubated for 45 min in TBST containing 5% dry skimmed milk and a sheep anti-rabbit peroxidase conjugate (Roche Molecular Biochemicals) diluted 1:3000. After another five washes of 10 min each in TBST, the blot was developed using the chemiluminescent system and according to the protocol supplied by Roche Molecular Biochemicals.

**Enzyme Assays**—Chorismate synthase activity was assayed using forward coupling of the reaction to anthranilate synthase at 30 °C essentially as described by Schaller et al. (7), except that the assay mixture used was 0.1 M potassium phosphate, pH 7.6, containing 30 mM ammonium sulfate, 10 mM glutamine, 4 mM magnesium sulfate, 1 mM diithiothreitol, 10 μM FMN, 50 picomol anthranilate synthase (from *E. coli*), and 80 μM ESPP. Either 5 mM sodium dithionite or 1 mM NADPH were used to start the reaction.

**CD Spectroscopy**—CD measurements were performed with a Jasco-715 spectropolarimeter equipped with a computer controlled water bath, using thermostatted cuvettes of 0.2-mm path length. Thermal shifts were followed by continuous monitoring of ellipticity at 222 nm between 3 and 92 °C at a scan rate of 1.0 degree min⁻¹ and with data collection every 20 s. Protein samples were prepared for CD spectroscopy in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate.

**PAGE**—Native PAGE was performed using the Pharmacia PhastSystem with an 8–25% gel run at pH 6.8 for 240 A² for 15 min at 15 °C. Either oxidized FMN and/or ESPP were added in 5-fold molar excess to samples of enzyme (15 μM, in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate) as indicated in the legend to Fig. 3 and 20 min before beginning electrophoresis. Gels were stained with Coomassie Blue. The protein standards used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (66 and 132 kDa).

**Analytical Ultracentrifugation**—Conventional sedimentation equilibrium measurements were made according to the method of Liu et al. (18) with a Beckman XL-A analytical ultracentrifuge (Beckman, Palo Alto, CA). The data were collected at 7,000, 9,000, and 12,000 rpm and at 20 °C. The sample volume was 120 μl at a protein concentration of 0.27 mg/ml in 10 mM Tris, pH 7.4, containing 90 mM potassium chloride. Protein samples were placed in the above buffer. The spectra were recorded with a Uvikon 933 spectrophotometer (Kontron Instruments AG, Zürich, Switzerland) equipped with a Haake D1 waterbath (Digitana AG, Horgen, Switzerland). The spectra were recorded in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate and by systematically varying either the substrate concentration or the temperature degree.

**UV-Visible Absorbance Spectrophotometry**—Absorbance spectra were recorded with a UVikon 933 spectrophotometer (Kontron Instruments AG, Zürich, Switzerland) equipped with a Haake D1 waterbath (Digitana AG, Horgen, Switzerland). The spectra were recorded in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate and by systematically varying either the substrate concentration or the temperature degree.

**Rapid Reaction Spectrophotometry**—Formation and decay of the flavin intermediate was observed using a stopped flow spectrophotometer equipped with a thermostatted 1-cm path length cell and a diode array detector (Spectroscopy Instruments GmbH, n=82205 Gilching) interfaced with a Macintosh Icx computer. Data were acquired using the chemiluminescent system and according to the protocol supplied by ZINS ZIEGLER-Instruments GmbH, Noberstrasse 3–5, D-41189 Mönchengladbach. Rapid reactions were recorded between 300 and 600 nm, the integration time for collecting a spectrum was 10 ms with a resolution of 2 pixels/nm. FMN was reduced with sodium dithionite in anaerobic solutions of the substrate and a stoichiometric concentration of enzyme. The experiments were performed in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate.

**RESULTS**

**PCR of the *aroC* Gene from *T. maritima***—Under the conditions used here, the polymerase chain reaction resulted in the amplification of a fragment which was ~1150 base pairs in size. Including the engineered restriction sites (19 bases), this fragment thus corresponds to the predicted size of the encoding *aroC* gene from *T. maritima* (1128 base pairs) based on the DNA sequence and was confirmed by DNA sequencing analysis.

Expression and Purification of Recombinant *T. maritima* Chorismate Synthase—The PCR product obtained was cloned...
into the expression vector pET21a to create the construct pET-TmaroC. Expression of *T. maritima* chorismate synthase (41.7 kDa) could be obtained in *E. coli* BL21(DE3) cells, as is the conventional protocol for this system (Fig. 1, panel A and B, lane 4), but greater expression could be achieved in *E. coli* BL21-CodonPlus™(DE3)-RIL cells (Stratagene) presumably due to rare codon usage in TmaroC for arginine and isoleucine residues (Fig. 1, panel A, lane 8). The expression of *T. maritima* chorismate synthase in BL21-CodonPlus™(DE3)-RIL cells is ~6-fold greater than that observed in BL21(DE3) (Fig. 1, panel B, compare protein loaded in lanes 4 and 8). However, expression in BL21-CodonPlus™(DE3)-RIL cells appears to be independent of induction (Fig. 1, panel B, lanes 7 and 8). The majority of the recombinant fusion protein obtained was insoluble (Fig. 2, lane 2). The soluble *T. maritima* chorismate synthase fraction (Fig. 2, lane 3) was refined in the first instance by a heat precipitation step which was optimal at 75 °C. At this temperature the majority of the contaminating proteins precipitated (Fig. 2, lane 4) and could be removed by centrifugation, while the *T. maritima* chorismate synthase remained in solution (Fig. 2, lane 5). The most prominent contaminating protein remaining at this stage was chloramphenicol acetyltransferase from *E. coli* (25.7 kDa), as determined by N-terminal amino acid sequencing (15 cycles of 50 pmol of protein resolved the amino acids MEKKITGYTTVDISQ, which are identical to the data base entry for this protein, accession number CAAD7774), and could be removed by chromatography using DEAE-Sephacel (Fig. 2, lane 6). The identity of the purified band as *T. maritima* chorismate synthase (41.7 kDa) was confirmed by N-terminal sequencing analysis (15 cycles of 50 pmol of protein resolved the amino acids MKLTIAGDSHGKYMV, which are identical to the data base entry for this chorismate synthase, accession number Q9WY12), and the protein was estimated to be greater than 95% pure, as judged from SDS-PAGE (Fig. 2, lane 6). A typical yield of *T. maritima* chorismate synthase under these conditions was 30 mg from 80 g of *E. coli* cell paste.

Requirement of *T. maritima* Chorismate Synthase for Reduced FMN—The purified recombinant *T. maritima* chorismate synthase is catalytically active (Table I) and its specific activity in the presence of dithionite and at 30 °C is calculated as 135.5 nmol-mg⁻¹·min⁻¹. The specific activity obtained with *T. maritima* chorismate synthase (Table I) is lower than that obtained with two mesophilic chorismate synthases (Table I, *N. crassa* and *E. coli*), assayed under the same conditions, which is at least partly due to nonoptimal temperature conditions for the thermophilic enzyme. While there was a 2-fold increase in the specific activity of *T. maritima* chorismate synthase on performing the reaction at 37 °C (data not shown), the instability of anthranilate synthase above 40 °C (the coupling enzyme used in this assay) did not permit determination of the optimal temperature of *T. maritima* chorismate synthase using this assay. In order to determine if *T. maritima* chorismate synthase is mono- or bifunctional, the enzyme assay was performed with either flavin reduced by dithionite exogenously, or adding NADPH to ascertain if the enzyme has the intrinsic ability to reduce the FMN cofactor itself as a measure of its monobifunctionality (where approaching 100% indicates a bifunctional enzyme). As the specific activity obtained for *T. maritima* chorismate synthase in the presence of NADPH is only 0.5% of that in the presence of dithionite (Table I, *T. maritima*), it can be concluded that it is monofunctional. The respective activities of the monofunctional *E. coli* chorismate synthase (Table I, *E. coli*) and the bifunctional *N. crassa* chorismate synthase (Table I, *N. crassa*) are indicated as controls.

**Table I**

| Source of protein | Flavin reductant | CS activity Relative* |
|-------------------|-----------------|----------------------|
| *T. maritima*     | Dithionite      | 136                   |
|                   | NADPH           | 0.7                  |
| *N. crassa*       | Dithionite      | 608                  |
|                   | NADPH           | 408                  |
| *E. coli*         | Dithionite      | 812                  |
|                   | NADPH           | 105                  |

* NADPH/dithionite CS activity × 100.

**FIG. 1.** Analysis of the expression of *T. maritima* chorismate synthase by SDS-PAGE. *A*, Coomassie Blue-stained gel; lanes 1–4 are the empty vector (pET-21a) and the vector containing *T. maritima* aroC (pET-TmCS), respectively, u = uninduced and i = induced (1 mM IPTG) and are as expressed in *E. coli* BL21(DE3) cells; lanes 5–8 are as for lanes 1–4 but in *E. coli* BL21-CodonPlus™(DE3)-RIL cells. The amount of protein loaded in each lane is ~6 μg. *TmCS, T. maritima* chorismate synthase. *B*, Western blot analysis of A using an antibody raised against *C. sempervirens* chorismate synthase. The panel A, Western blot analysis of A using an antibody raised against *C. sempervirens* chorismate synthase. The amount of protein loaded in lanes 1–4 is ~0.6 μg while in lanes 5–8 it is ~0.1 μg.

**FIG. 2.** Analysis of the purification of *T. maritima* chorismate synthase by SDS-PAGE. Lane 1, molecular mass markers as indicated; lane 2, pellet of crude cell extract after centrifugation; lane 3, supernatant of crude cell extract after centrifugation; lane 4, precipitated protein after heat treatment; lane 5, soluble protein after heat treatment; lane 6, purified *T. maritima* chorismate synthase (TmCS) after DEAE-Sephacel chromatography.
Determination of the Melting Temperature of Chorismate Synthase in the Presence and Absence of Ligands—The stability of both *E. coli* and *T. maritima* chorismate synthase in the presence and absence of both oxidized FMN and EPSP was examined using temperature unfolding experiments as measured by CD spectroscopy (Fig. 3). The melting temperatures of *E. coli* chorismate synthase in the absence and presence of ligands (54.7 ± 0.5 °C and 58.9 ± 0.04 °C, respectively) indicate a stabilizing effect of the ligands (Fig. 3, A-C). The melting temperature of *T. maritima* chorismate synthase was estimated to be a minimum of 92 °C in both the presence and absence of ligands (Fig. 3, D-F). However, the difference (if any) of the melting temperature for this enzyme with and without ligands could not be determined from these experiments.

Native PAGE of Chorismate Synthase in the Presence and Absence of Ligands—Chorismate synthase from *E. coli* has been reported to be a homotrimer (156 kDa) and when run on native-PAGE (8–25%) appears as a diffuse band with a mobility corresponding to an apparent mass of ~190 kDa (19). When the enzyme is preincubated in the presence of both oxidized FMN and EPSP and subjected to native-PAGE as for the enzyme alone, there is a marked shift in the mobility toward the anode (140–150 kDa), which does not occur in the presence of either substrate alone (19). The same analysis was performed here with *T. maritima* chorismate synthase and its properties were compared with those of the *E. coli* enzyme (Fig. 4). In contrast to the *E. coli* enzyme (Fig. 4, *E. coli* chorismate synthase, lane 1), *T. maritima* chorismate synthase alone appears as a sharp band with a mobility corresponding to ~200 kDa (Fig. 4, *T. maritima* chorismate synthase, lane 1). However, when *T. maritima* chorismate synthase is incubated in the presence of both oxidized FMN and EPSP, there is a slight shift in mobility toward the anode akin to what was observed with the *E. coli* enzyme but not nearly as pronounced (Fig. 4, compare lane 4 of *E. coli* and *T. maritima* chorismate synthase). Similar to what was observed with the *E. coli* enzyme, there is no apparent shift in the mobility of *T. maritima* chorismate synthase when the enzyme is preincubated with either of the substrates alone (Fig. 4, compare lanes 2 and 3 of *E. coli* and *T. maritima* chorismate synthase, respectively).

Determination of the Quaternary Structure of Chorismate Synthase—The molecular mass of *T. maritima* chorismate synthase was estimated by performing sedimentation equilibrium experiments at a variety of operational speeds in an analytical ultracentrifuge. A representative data set from a sedimentation equilibrium experiment is shown in Fig. 5. The data can be interpreted as resulting from a single ideal species according to the model of Liu et al. (18) under all the conditions used. The molecular mass of the enzyme was calculated to be 168,377 ± 1950 Da under each of the conditions used. The molecular mass of the monomer estimated from the amino acid sequence is 41,754 Da. In addition, the enzyme prepared here gave a single homogenous band on SDS-PAGE with a molecular mass of ~42 kDa. Therefore, it can be concluded that *T. maritima* chorismate synthase is a tetramer of identical subunits.

UV-Visible Absorption Spectral Properties of *T. maritima* Chorismate Synthase—Chorismate synthase isolated from *T. maritima* under the conditions used here, showed a single absorption maximum at 278 nm (Fig. 6, panel A). There were no absorbance maxima characteristic for bound flavin (i.e. ~370 nm and ~450 nm), thus indicating that chorismate synthase is isolated as the apoenzyme form. Oxidized free FMN (25 μM in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate) exhibits absorption maxima at 372 and 445 nm, respectively (Fig. 6, panel A). Addition of 28 μM chorismate synthase to 25 μM oxidized FMN at room temperature caused no perturbation of these maxima (Fig. 6, panel A). Subjecting this solution to ultracentrifugation (Centricon 30) allowed the estimation of a *K*D of 137 μM for binding of oxidized FMN to the enzyme at room temperature (Table II). This result indicates the weak binding of the oxidized co-factor under these conditions and accounts for the isolation of the protein in the apoenzyme form and is akin to what has been observed with *E. coli* chorismate synthase (20). A gradual increase of the temperature of the chorismate synthase-oxidized FMN solution to 66 °C led to a hypochromic shift of the absorption maximum at 372 to 360 nm and was accompanied by a hypochromic effect on the absorption intensity in this region (Fig. 6, panel B). Albeit to a lesser degree, the increase in temperature also produced an increase in the resolution of the absorption maximum around 450 nm and induced a slight hypochromic shift of the absorption maximum in this region from 445 to 442 nm (Fig. 6, panel B). The *K*D for the binding of oxidized FMN to the chorismate synthase after this heating step was estimated to be 3 μM, indicating 46-fold tighter binding.

The addition of EPSP to the heated chorismate synthase/chorismate synthase (5 μM) in the absence of ligands.

**Fig. 3.** Comparison of the thermal unfolding of *E. coli* and *T. maritima* chorismate synthases in the presence and absence of ligands. A, *E. coli* chorismate synthase (5 μM) in the presence of ligands; B, *E. coli* chorismate synthase (5 μM) in the presence of oxidized FMN (5 μM) and EPSP (10 μM); C, overlay of A and B. D, *T. maritima* chorismate synthase (5 μM) in the presence of oxidized FMN (5 μM) and EPSP (10 μM). E, *T. maritima* chorismate synthase (5 μM) in the absence of ligands; F, overlay of D and E. All experiments were carried out in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate. The melting temperatures were determined from the best fit of the data to the sigmoidal function f(x)= (a-d/1 + (ucx)^f) + d, where c = the value at the inflection point.

Temperature (degrees Celsius)
FMNox solution causes spectral changes which are predominantly manifested in the 450-nm absorption maximum only (Fig. 6, panel B). The main effect is an amplified resolution of the absorption maximum in this region compared with the absorption maximum in the visible region of the spectrum, respectively (Fig. 7). From this figure it can be seen that the temperature increase (oxidized FMN, 25 μM) and the difference spectra amplified between 300 to 600 nm. B, absorbance spectrum of 25 μM oxidized FMN at 28 °C (—), after heating to 66 °C ( — — — ). The inset shows the absorbance changes at 383 nm as a function of the concentration of EPSP, showing the absorbance changes at 383 nm as a function of the concentration of EPSP.

**TABLE II**

Dissociation constants (K_D) of T. maritima chorismate synthase for oxidised FMN and EPSP at 28 °C

| Treatment     | K_D FMN | K_D′ FMN | K_D EPSP |
|---------------|---------|----------|----------|
| Untreated     | 137     | 99       | 3        |
| Heated        | 3       | 2        | 0.3      |

K_D of oxidized FMN in the presence of EPSP.
K_D EPSP in the presence of oxidised FMN.
K_D after heating to 66 °C and cooling down to 28 °C.

FMNox from 28 to 66 °C produced a slight decrease in the intensity of the absorption maxima at 372 and 445 nm (data not shown), but the changes were insignificant compared with those observed in the presence of the protein and EPSP. The presence of EPSP slightly decreases the K_D of oxidized FMN at 28 °C and after the heat treatment, respectively (Table II). We also estimated an apparent K_D for the binding of EPSP to the enzyme in the presence of oxidized FMN from the absorbance changes at 383 nm as a function of the concentration of EPSP (Fig. 6, panel B, inset). The apparent K_D estimated for EPSP (3 μM) from these experiments decreases 10-fold upon heat treatment of the enzyme (Table II).

**Characterization of the Flavin Intermediate**—In 1991 Ramjee et al. (21) reported on the detection (using stopped-flow
spectrophotometry) of a transient flavin intermediate formed during the catalytic cycle of E. coli chorismate synthase and exclusively associated with the binding of the substrate EPSP. Further studies showed that this intermediate occurs before the substrate EPSP is actually consumed (i.e. before C-O and C-H bond cleavage) (22). Complementary studies showed that in the absence of substrate and under the conditions used, pH 7.0, the reduced flavin is bound to the protein in its deprotonated or monoanionic state (pK_a = 6.7) (20), while in the presence of substrate the reduced flavin is bound in its protonated or neutral form (23). Hence, the occurrence of the flavin intermediate reflects the protonation of the deprotonated reduced flavin upon binding of substrate. As this has been one of the key observations in the investigation of the mechanism of chorismate synthase, we performed the same study here using rapid reaction spectrophotometry and the T. maritima enzyme.

The difference in absorption observed between a solution of chorismate synthase/FMNred in the presence and absence of EPSP; difference spectrum between chorismate synthase/oxidized FMN at 66 °C in the presence and absence of EPSP; difference spectrum between chorismate synthase/oxidized FMN/EPSP at 66 °C and that at 28 °C; D, composite of A + B.

**Fig. 7.** Difference absorbance spectra of T. maritima chorismate synthase. A, difference spectrum between chorismate synthase (28 μM) and oxidized FMN (25 μM) at 66 °C and 28 °C; B, difference spectrum between chorismate synthase/oxidized FMN at 66 °C in the presence and absence of EPSP; C, difference spectrum between chorismate synthase/oxidized FMN/EPSP at 66 °C and that at 28 °C; D, composite of A + B.

**DISCUSSION**

To our knowledge, chorismate synthase has not been purified to homogeneity and characterized from any extremophilic organism until now. Characterization of the enzyme was aided by cloning and expressing the enzyme in an E. coli system which resulted in a yield of T. maritima chorismate synthase suitable to enable the observations reported in this study. The purification procedure is quick and relatively easy, the greatest refinement being achieved by the heat treatment step at 75 °C which removes almost 80% of contaminating proteins as judged from SDS-PAGE analysis. The monofunctionality of T. maritima chorismate synthase from the evolutionary standpoint of chorismate synthases is very interesting. While it has been concluded from an earlier phylogenetic analysis that chorismate synthases are monophyletic (12), it is not known to date if the ancestral chorismate synthase is mono- or bifunctional. However, it has been suggested that the common ancestor was probably bifunctional given that it is difficult to imagine the evolution of the intrinsic reductase activity in a framework of monofunctional enzymes (12). It was surmised that bifunctionality may have either been maintained only in organisms in which the availability of reduced flavin is limiting or perhaps there was positive selection of monofunctionality (12). T. maritima is thought to be one of (if not) the oldest eubacterium and appears to have undergone considerable lateral gene transfer from the archaea (14). A phylogenetic tree of all chorismate synthases presently known (data not shown) suggests that T. maritima chorismate synthase diverged with the archaea and moreover, considerably before any of the chorismate synthases for which bifunctionality is known (i.e. N. crassa and Saccharomyces cerevisiae). Thus the classification of its chorismate synthase as monofunctional could be considered to be cognate to the ancestral chorismate synthase and would therefore not lend support to bifunctionality being ascendant.

This is the first report on the thermal denaturation behavior

**Fig. 8.** Single turnover experiment with T. maritima chorismate synthase. An anaerobic solution of chorismate synthase (65 μM) and FMN (65 μM, reduced with excess sodium dithionite) was mixed with EPSP (52 μM) in the stopped-flow instrument. The spectra show the difference in absorbance detected in the presence and absence of EPSP and were recorded at the times indicated. The inset shows the absorbance changes at 390 nm as a function of time. The experiment was performed at 25 °C in 10 mM HEPES, pH 7.4, containing 50 mM ammonium sulfate.
of any chorismate synthase. For the mesophilic E. coli enzyme the 4 °C increase in the melting temperature in the presence of ligands appears to be related to the major conformational change in E. coli chorismate synthase in the presence of FMN and EPSP to a more compact structure (19). As the melting temperature of T. maritima chorismate synthase could only be estimated under the conditions used here it was not possible to ascertain the effect (if any) on ligand binding. However, obviously and as would be expected, the melting temperature (92 °C) is higher than the optimal temperature for growth of the organism (80 °C).

The quaternary structure of T. maritima chorismate synthase is represented by a tetramer of identical subunits. This appears to “fit” with the known quaternary structures of other chorismate synthases in that the hierarchy is spread between that of dimer-tetramer (12). Interestingly at the quaternary level, a remarkable feature of hyperthermophiles is the occurrence of anomalous states of association and fused multifunctional proteins (24). However, even though there are many examples for these features (24), T. maritima chorismate synthase does not appear to be one of them. Therefore, additional quaternary interactions cannot account for the higher stability of this protein. This question may be answered in the future by performing specific mutations in the contact surface of the subunits to establish their contribution to the quaternary structure.

From the native-PAGE studies performed here it could be concluded that the apoprotein from T. maritima appears to have a higher structural rigidity compared with that of the E. coli apoprotein which is reflected by the sharp band of the former compared with the rather diffuse band of the latter on native-PAGE. The change in the mobility of E. coli chorismate synthase in the presence of ligands has previously been interpreted to reflect less conformational flexibility than that of the apoprotein (19). In support of this, the decrease in mobility of T. maritima chorismate synthase in the presence of ligands is not as pronounced as that observed with the E. coli enzyme which may reflect the lower flexibility of the thermophilic apoprotein compared with that of the mesophilic enzyme.

In aqueous solution, the near UV-visible absorption spectra of free flavins exhibit two featureless bands at about 450 and 375 nm (25). However, in solvents less polar than water the band at 375 nm shifts to a shorter wavelength and the visible band at about 450 nm shows greater resolution with two pronounced shoulders which are thought to be associated with vibrational transitions in the first electronic absorption at this wavelength (26, 27). Additionally, it has been shown that the spectra of many flavoproteins are akin to that observed for free flavin in an apolar solvent indicating such an environment of the flavin cofactor when bound to the protein. Importantly, most flavoproteins show a shift in the 370 nm region to a shorter wavelength and a varying resolution of the band at about 450 nm (25). With T. maritima chorismate synthase, an apolar type flavin spectrum is observed when the temperature of the protein/FMN solution is increased, thus reflecting induced FMN binding. This is borne out by the fact that the K_D for FMN binding is ~46-fold lower after heating to 66 °C and cooling to 28 °C compared with that measured at 28 °C without heating to 66 °C (3–137 μM, respectively). Upon FMN binding, the spectral transition is mainly manifested in the 370-nm region which is consistent with solvent-dependent spectral shifts (more apolar) with little development of fine structure. This is accompanied by slightly better resolution in the 450-nm region which indicates that the vibronic transitions in this area are more discrete. This is clearly due to binding of the flavin to the protein which reduces the rotational freedom of the flavin and possibly introduces some strain on the bound cofactor. The phenomenon is further enhanced by the presence of EPSP which substantially increases the band resolution in this area and thus probably the constraint on the flavin. This observation may reflect that T. maritima chorismate synthase has a more rigid/compact structure at room temperature which upon heating becomes more flexible resulting in an increase in the rate of ligand binding and in fact probably allows the cofactor to enter into the active site. This view is supported by the fact that cooling the protein/FMN solution to room temperature after heating does not reverse the process, indicating that the FMN is now “trapped” in the active site.

The flavin-derived transient intermediate previously observed for the E. coli enzyme (21) was also observed in this study with T. maritima chorismate synthase. As for the E. coli enzyme, formation of this flavin species was associated with binding of the substrate, i.e. formation of a ternary complex between enzyme, reduced flavin, and EPSP. Formation of this complex is considered to reflect protonation of the anionic reduced flavin, at least for the E. coli enzyme (20, 23). This protonation of the reduced flavin indicates that the flavin experiences a different polarity in the active site when substrate binds to the enzyme and is supported by the apolar-type flavin spectra observed here when EPSP is added to T. maritima chorismate synthase/FMNox after heating. The observation suggests that the catalytic action of T. maritima chorismate synthase proceeds with a mechanism similar to that of E. coli chorismate synthase, i.e. flavin-derived intermediate formation (reduced FMN in neutral form) precedes C-O and C-H bond cleavage of EPSP (22).

Finally, one of the major drawbacks at present in proceeding with studies on this intriguing enzyme is the lack of a three-dimensional structure. For x-ray crystallography, it has been suggested that adequately diffracting crystals can be obtained more readily with thermophilic enzymes due to their greater rigidity and stability (28). The apparent rigidity and stability of the protein described here coupled with the characteristics of substrate binding make it an exceptional source with regard to the possibility of elucidating the structure of chorismate synthase.

Acknowledgment—We thank Sandro Ghisla for allowing us to use the stopped-flow spectrophotometer at the University of Konstanz.

REFERENCES

1. Hill, R. K., and Newkome, G. R. (1969) J. Am. Chem. Soc. 91, 5893–5894
2. McNamee, P. J., Miller, G., and Coggins, J. R. (1988) Biochem. J. 251, 313–322
3. Morel, H., Clark, M. J., Knowles, P. F., and Sprinson, D. B. (1987) J. Biol. Chem. 262, 82–90
4. Welch, G. R., Cole, K. W., and Gaertner, F. H. (1974) Arch. Biochem. Biophys. 165, 505–518
5. White, P. J., Miller, G., and Coggins, J. R. (1988) Biochem. J. 251, 313–322
6. Moussallem, D. M., and Coggins, J. R. (1986) FEBS Lett. 205, 328–332
7. Schaller, A., Windhoefer, V., and Amrhein, N. (1990) Arch. Biochem. Biophys. 282, 437–442
8. Schaller, A., Schmid, J., Leibinger, U., and Amrhein, N. (1991) J. Biol. Chem. 266, 21434–21438
9. Schaller, A., von Afferden, M., Windhoefer, V., Bilow, S., Abel, G., Schmid, J., and Amrhein, N. (1991) Plant Physiol. 97, 1271–1279
10. Schaller, A., Cole, K. W. (1973) J. Biol. Chem. 248, 4602–4609
11. Schaller, F. H. (1987) Methods Enzymol. 142, 362–366
12. Schaller, A., Schmid, J., Amrhein, N., and Schaller, A. (1999) Planta 207, 325–334
13. Nelson, K. R., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., Biech, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton, M. D., Pratt, M. S., Phillips, C. A., Richardson, D., Heidelberg, J., Sutton, G. G., Fleischmann, R. D., Eisen, J. A., White, O., Salzberg, S. L., Smith, H. O., Venter, J. C., and Fraser, C. M. (1999) Nature 399, 323–329
14. Achenbach-Richter, L., Gupta, R., Stetter, K., and Woese, C. (1987) Syst. Appl. Microbiol. 9, 34–39
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, A. J., and Struhl, K. (1987) Current Protocols in Molecular Biology, Greenw Publishing Associates and Wiley Interscience, New York
16. Bambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A
17. Laemmli, U. K. (1970) *Nature* **227**, 680–685
18. Liu, N., Deillon, C., Klaus, S., Gutte, B., and Thomas, R. (1998) *Protein Sci.* **7**, 1214–1220
19. Macheroux, P., Schirbrun, E., Svergun, D. I., Volkov, V. V., Koch, M. H. J., Bornemann, S., and Thorneley, R. N. F. (1998) *Biochem. J.* **335**, 319–327
20. Macheroux, P., Petersen, J., Bornemann, S., Lowe, D. J., and Thorneley, R. N. F. (1996) *Biochemistry* **35**, 1643–1652
21. Ramjee, M. K., Coggins, J. R., Hawkes, T. R., Lowe, D. J., and Thorneley, R. N. F. (1991) *J. Am. Chem. Soc.* **113**, 8566–8567
22. Bornemann, S., Lowe, D. J., and Thorneley, R. N. F. (1996) *Biochemistry* **35**, 9907–9916
23. Macheroux, P., Bornemann, S., Ghisla, S., and Thorneley, R. N. F. (1996) *J. Biol. Chem.* **271**, 25850–25858
24. Jaenicke, R., and Böhm, G. (1998) *Curr. Opin. Struct. Biol.* **8**, 738–748
25. Müller, F., Mayhew, S. G., and Massey, V. (1975) *Biochemistry* **12**, 4654–4662
26. Harbury, H. A., La Neve, K. F., Leach, P. A., and Amick, R. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **45**, 1708–1717
27. Weber, G. (1966) in *Flavins and Flavoproteins* (Slater, E. C., ed) pp. 15–21, Elsevier, Amsterdam
28. Dams, T., and Jaenicke, R. (1999) *Biochemistry* **38**, 9169–9178