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ORIGINAL PAPER

Characterization of a key trifunctional enzyme for aromatic amino acid biosynthesis in *Archaeoglobus fulgidus*

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Abstract In the aromatic amino acid biosynthesis pathway, chorismate presents a branch point intermediate that is converted to tryptophan, phenylalanine (Phe), and tyrosine (Tyr). In bacteria, three enzymes catalyze the conversion of chorismate to hydroxyphenylpyruvate or pyruvate. The enzymes, chorismate mutase (CM), prephenate dehydratase (PDT), and prephenate dehydrogenase (PDHG) are either present as distinct proteins or fusions combining two activities. Gene locus AF0227 of *Archaeoglobus fulgidus* is predicted to encode a fusion protein, AroQ, containing all three enzymatic domains. This work describes the first characterization of a trifunctional AroQ. The *A. fulgidus aroQ* gene was cloned and overexpressed in *Escherichia coli*. The recombinant protein purified as a homohexamer with specific activities of 10, 0.51, and 50 U/mg for CM, PDT, and PDHG, respectively. Tyr at 0.5 mM concentration inhibited PDHG activity by 50%, while at 1 mM PDT was activated by 70%. Phe at 5 μM inhibited PDT activity by 66% without affecting the activity of PDHG. A fusion of CM, PDT, and PDHG domains is evident in the genome of only one other organism sequenced to date, that of the hyperthermophilic archaeon, *Nanoarchaeum equitans*. Such fusions of contiguous activities in a biosynthetic pathway may constitute a primitive strategy for the efficient processing of labile metabolites.

Keywords Chorismate mutase · Prephenate dehydrogenase · Prephenate dehydratase · Evolution · Archaea · *Archaeoglobus fulgidus* · Aromatic amino acid biosynthesis

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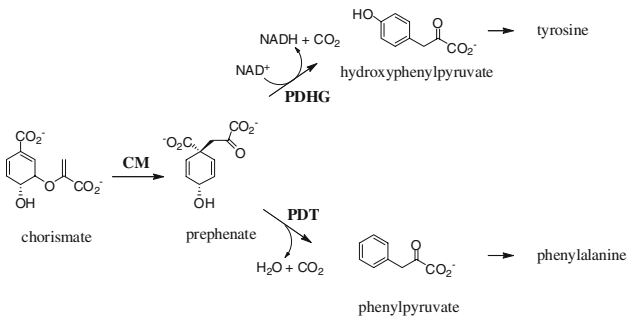
Introduction

Chorismate is the first committed intermediate in the biosynthesis of phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). The shikimate pathway leading to the formation of chorismate is commonly found in archaea, bacteria, fungi, and plants. Chorismate mutase (CM; EC 5.4.99.5) catalyzes the conversion of chorismate to prephenate, which is either decarboxylated to phenylpyruvate by prephenate dehydratase (PDT; EC 4.2.1.51) or oxidized/decarboxylated to hydroxyphenylpyruvate by NAD^+-dependent prephenate dehydrogenase (PDHG; EC 1.3.1.12). Subsequent reactions lead to the formation of Phe and Tyr, respectively. In all organisms studied thus far, two or more independent enzymes catalyze the generation of phenylpyruvate and hydroxyphenylpyruvate from chorismate.



Chorismate mutase enzymes fall into two classes: monofunctional AroH-type enzymes forming an α/β-barrel structure, and AroQ-type enzymes, which are helix-bundle proteins that can harbor additional catalytic domains (Dosselaere and Vanderleyden 2001). The $AroQ_p$ enzymes consist of a CM domain fused to a PDT domain, while the $AroQ_t$ enzymes comprise a fusion of the CM domain to a PDHG domain. In the $AroQ_d$ enzymes the CM domain is fused to a 2-dehydro-3-deoxyphosphoheptonate aldolase (DAHP synthase). $AroQ_f$ and $AroQ_r$ are monofunctional CMs that are unregulated and allosterically regulated by the end products of the aromatic amino acid biosynthesis pathway (Phe, Tyr, or Trp), respectively.

Aromatic amino acids are of particular interest due to their potential roles as intermediates in the synthesis of many industrially important products, such as aspartame, melanin, and indigo. As such, enzymes utilizing the branch point intermediate, chorismate, have been explored extensively in bacteria (Koch et al. 1970, 1971; Friedrich et al. 1976; Xia et al. 1993; Zhang et al. 1998; Helmstaedt et al. 2004; Sasso et al. 2005). In archaea, characterization of this pathway is limited to studies of enzymes from *Methanococcus* and *Methanocaldococcus* species (MacBeath et al. 1998; Porat et al. 2004; Kleeb et al. 2006).

In this study, we describe the characterization of the *Archaeoglobus fulgidus* AroQ which represents a new fusion protein combining CM, PDT, and PDHG activities. *A. fulgidus* is a marine sulfate reducer that grows optimally at 83°C. The physiological implications for this trifunctional enzyme and its phylogenetic relationship to closely related proteins are discussed.

Materials and methods

Strains, plasmids, growth conditions, and DNA manipulations

Escherichia coli strain BL-21-CodonPlus® (DE3)-RIL (Stratagene) was used for gene overexpression. The strain was cultured in LB medium containing chloramphenicol at 0.05 g/l and ampicillin at 0.1 g/l when transformed with the pIS85 expression plasmid. For gene expression, bacteria were grown in 3-l flasks containing 1 l of LB under constant shaking at 37°C. Gene expression was induced by the addition of 0.4 mM IPTG to a culture at OD_{600} of 0.6, and the biomass was harvested 2 h after induction by centrifugation (Sorval RC2B) at 6,000 × *g* for 10 min.

Plasmid pIS85 was constructed by PCR cloning the *aroQ* gene from *A. fulgidus* DSM4304 chromosomal DNA using the following primers: 5′CCTGGACATATGATTTC TGTCAAGATTC3′ and 5′CTAGAGGATCCTTACTAGA GGGTGCTCATCCGCTTGACCTCATC3′. In the forward primer, the GTC start codon on the genome was modified to ATG to facilitate translation in *E. coli*.

Crude genomic *A. fulgidus* DNA was prepared by removing 1 ml of *A. fulgidus* culture (OD_{500} of 0.5), which was sedimented by centrifugation at 3,000 × *g* for 10 min and resuspended in 1 ml deionized water. The cell suspension was sheared twice by passing it through a 23-gauge needle. Five microliter of the cell suspension was used directly in the PCR reaction. The amplified DNA fragment was digested with *Nde*I and *Bam*HI (restriction enzyme sites are underlined in the primers) and cloned into pET-3a (Novagen). Wildtype DNA sequence was confirmed by DNA sequence analysis (Laragen). DNA manipulations and transformation procedures were done according to standard procedures (Perbal 1988).

Protein purification

All purification steps were performed at room temperature unless noted otherwise. The frozen cell pellet was resuspended in 20 mM PIPES, pH 7.0, 1 mM $MgCl_2$, and DNase and RNase were added at 10 μg/ml. The cell suspension was passed three times through a French pressure cell (Thermo Scientific) at 16,000 psig. The membrane fraction was removed by ultracentrifugation at 200,000 × *g* for 1 h. The soluble fraction was heat-treated by incubating at 80°C for 20 min in the presence of 1 M NaCl. The addition of 1 M NaCl stabilized the protein during the 20-min heat treatment. Precipitated *E. coli* protein was removed by centrifugation at 32,000 × *g* for 10 min. The remaining soluble protein solution was applied to a 20-ml Phenyl Sepharose hydrophobic interaction column (GE Healthcare) equilibrated with 0.5 M $(NH_4)_2SO_4$ in 20 mM PIPES, pH 7.0. Fractions containing CM/PDT/PDHG activity eluted at zero salt concentration and were concentrated using a Millipore Centriplus 50,000 molecular-weight-cut-off (MWCO) centrifuge filter. Highly purified protein was obtained by applying concentrated phenyl sepharose eluent to a 1-ml Q Sepharose anion exchange column (GE Healthcare) equilibrated with 20 mM Bis–Tris, pH 6.5, containing 150 mM NaCl. The bulk of the enzyme was eluted in a 230–400 mM NaCl

gradient. The concentrated, purified enzyme was stored at −80°C in the elution buffer.

Molecular weight determination

The apparent molecular weight of the CM/PDT/PDHG enzyme was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the following protein standards (Sigma, SDS-7): Bovine serum albumin (M_r 66,000), chicken ovalbumin (M_r 45,000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), bovine carbonic anhydrase (M_r 29,000), bovine pancreas trypsinogen (M_r 24,000), soybean trypsin inhibitor (M_r 20,100), and bovine milk α-lactalbumin (M_r 14,200). The apparent molecular weight of the native protein was determined by gel filtration chromatography using a Superdex 200 10/30 column (GE Healthcare) with bovine serum albumin (67,000), catalase (232,000), ferritin (440,000), thyroglobulin (669,000) as the standards, and blue dextran (2,000,000) to determine void volume. The column was equilibrated with 150 mM NaCl in 50 mM sodium phosphate buffer, pH 7.0, and was operated at flow rate of 0.4 ml/min. All samples were filtered (0.2 μm) prior to injection.

Protein and enzyme assays

The protein concentration was measured using the Bradford method (Bio-Rad) with bovine serum albumin as the standard.

Prephenate dehydrogenase activity was assayed at 87°C by following the reduction of NAD^+ at 340 nm ($\varepsilon_{NADH} = 6.22\ mM^{-1}\ cm^{-1}$) according to an assay modified from Koch et al. (1970). The assay mixture contained 2 mM barium prephenate, 0.5 mM NAD^+ in 50 mM MOPS, pH 7.5 (titrated at 25°C). Due to the pH change of MOPS buffer at elevated temperature, the actual pH at 87°C was calculated to be 6.8. After a 2-min pre-incubation at 87°C the reaction was started by the addition of enzyme. The reaction was run for 2 min.

Chorismate mutase activity was determined indirectly by following the associated PDHG activity spectrophotometrically. Using spectroscopy we determined that chorismate degrades chemically approximately by 30% in 5 min at 87°C under the assay conditions and, therefore, we performed the assay at 60°C where chemical degradation was reduced. The assay mixture was as described above but the solution contained 2 mM chorismate in place of prephenate. In addition, CM activity was determined directly by following the decrease of chorismate at 274 nm ($\varepsilon_{chorismate} = 2.63\ mM^{-1}\ cm^{-1}$) in a procedure adapted from Heyde and Morrison (1978). The assay mixture contained approximately 0.8 mM chorismate in 100 mM MES, pH 6.5, or 100 mM tricine, pH 8 (titrated at 25°C). The corresponding pH values at 60°C were 6.1 and 7.2, respectively. The reaction was started by addition of 2.4 μg enzyme.

Prephenate dehydratase activity was detected qualitatively using liquid chromatography/mass spectrometry (LC/MS) and quantitatively using a spectrophotometric assay. For the qualitative assay, the enzyme reaction was performed in microcentrifuge tubes containing 1 mM prephenate in water due to buffer interference in the LC/MS equipment. Following pre-incubation for 2 min at 87°C, the reaction was initiated by addition of enzyme. A control reaction was performed in the absence of enzyme. Formation of phenylpyruvate was monitored by LC/MS at the characteristic 164-Da peak. PDT activity was also quantified by measuring the appearance of phenylpyruvate at 320 nm ($\varepsilon_{phenylpyruvate} = 7.00\ mM^{-1}\ cm^{-1}$). The spectrophotometric PDT assay was performed at 77°C and the mixture contained 1 mM prephenate in 200 mM Tris buffer at several different pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5; Cotton and Gibson 1965). Following pre-incubation, the reaction was started by addition of enzyme and quenched by addition of 1 M NaOH with subsequent chilling on ice. The samples were centrifuged to remove precipitated protein, and the final absorbance was measured. To obtain kinetic parameters, the reaction was stopped at different time points. Each assay was done in triplicate.

Each respective activity calculation was corrected for abiotic prephenate or chorismate degradation. Unit of activity was defined as μmol of substrate consumed or product formed per minute at the given temperature. All chemicals were purchased from Sigma. The purity of the chorismate and prephenate, both as the barium salt, was 60–80% and 87%, respectively.

Gel electrophoresis

SDS-PAGE was performed with 20% homogeneous polyacrylamide gels (GE Healthcare) or with 4–20% gradient polyacrylamide gels (Invitrogen). The protein was preincubated in loading buffer at 90°C for 10 min.

Liquid chromatography/mass spectrometry

The presence of substrate and/or product was determined qualitatively utilizing the Waters LC/MS system (Thermabeam Mass Detector with 2,695 separation module and 996 photodiode array detector). The reverse-phase column was YMC ODS-AQ 3 μm (2.0 mm × 150 mm). The liquid mobile phase was acetonitrile:water (50:50) and was run at 0.2 ml/min. The temperatures of the nebulizer,

expansion region, and ionizers were set at 75, 80, and 210°C, respectively.

Phylogenetic tree

Amino acid sequences homologous to the *A. fulgidus* AroQ were identified using BLASTp databases. The construction of the phylogenetic tree was based on amino acid sequences of PDT protein available in the ExPASy database (Expert Protein Analysis System, Swiss Institute of Bioinformatics, http://ca.expasy.org/). Homologous amino acid sequences were aligned with the ClustalW program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) using Multialign (Corpet 1988). For database accession numbers, see the legend to Fig. 5. An unrooted phylogenetic tree was constructed using the neighbor-joining algorithm of PHYLIP (Phylogeny Inference Package, v 3.5c; Felsenstein 1993).

Results and discussion

Overexpression and purification

AroQ is a key enzyme of the aromatic amino acid biosynthesis pathway. The *A. fulgidus* AroQ appeared to be particularly interesting since its gene at locus AF0227 is predicted to encode a protein containing three domains harboring putative CM, PDT, and PDHG activities (MacBeath et al. 1998). In bacteria, proteins with only one or two of these domains are present. To confirm and characterize the putative trifunctional *A. fulgidus* AroQ, its gene was overexpressed in *E. coli*. An attempt to heat-purify the recombinant AroQ from *E. coli* cell extract in the absence of salt resulted in precipitation of AroQ. However, addition of 1 M NaCl stabilized the enzyme retaining approximately 50% of the PDHG activity. Salt stabilization of hyperthermophilic archaeal enzymes has been described previously (Lim et al. 2004; Schröder et al. 2004). *A. fulgidus* AroQ was purified to homogeneity by hydrophobic interaction and anion exchange chromatography. The final yield as determined using PDHG activity was approximately 13%. Further purification optimization was not performed since our goal was to obtain pure protein for characterization purposes.

The molecular weight of the purified enzyme was estimated to be 66,000 using SDS-PAGE analysis, while the calculated molecular weight was 70,946. The lower than expected molecular weight determined by SDS-PAGE is likely due to incomplete unfolding of the thermostable protein and was not investigated further. Size exclusion chromatography of the native enzyme indicated a total molecular weight of 420,000 suggesting a homohexameric structure. The *A. fulgidus* AroQ enzyme is of unusually large size compared to other archaeal or bacterial AroQs which typically form dimers or tetramers (Davidson et al. 1972; Xia et al. 1993; Euverink et al. 1995; MacBeath et al. 1998). An exception is the 210 kDa, oligomeric PDT from *Bacillus subtilis* (Riepl and Glover 1978). This enzyme assembles as a homo-octamer in the presence of its effector, methionine, and dissociates to a dimer at 0.1 mM concentration of Phe. In the absence of any effector, the enzyme dissociates into monomers with subsequent loss of activity. Ligand-induced oligomerization as a form of allosteric control has also been observed for the CM/PDT enzyme from *Salmonella typhimurium*, which dimerizes in the presence of its feedback inhibitor, Phe (Schmit and Zalkin 1971). The effect of ligands or salt on the oligomerization of the *A. fulgidus* AroQ enzyme as a possible feature of regulation has yet to be investigated.

Estimation of kinetic and thermodynamic parameters

The *A. fulgidus* AroQ was assayed for the CM, PDT, and PDHG activities using modified procedures. A problem with the assays was the inherent instability of the substrates. Chorismate was reported to undergo complete chemical decomposition into *p*-hydroxybenzoic acid and prephenate within 1 h at 70°C and pH 10 (Dawson et al. 1989). Free prephenic acid decomposed completely into phenylpyruvic acid and CO_2 within 10 min at 37°C in 0.5 M HCl, while no detectable loss was observed at pH 6.8. However, upon heating, phenylpyruvic acid formed *p*-hydroxyphenyllactic acid (Dawson et al. 1989). Because of the apparent heat lability of the substrates, kinetic values were corrected for degradation at high temperature. Direct quantification of prephenate and chorismate degradation using LC/MS was difficult due to the high impurities of the starting material and indistinguishable ionization products. However, significant end product concentrations were generated in the course of the enzyme assays to confirm the CM, PDT, and PDHG activities of the enzyme.

Prephenate dehydrogenase specific activity was measured to be 50 U/mg at 87°C. The K_m values for prephenate and NAD^+ were estimated at 0.45 ± 0.03 and 0.047 ± 0.001 mM, respectively. The enzyme exhibited a k_{cat}/K_m value for prephenate of 8.8×10^5 $M^{-1}s^{-1}$. Activity with $NADP^+$ as coenzyme was $\sim$ 10% of that with NAD^+, suggesting that NAD^+ is likely the preferred and physiological coenzyme. This preference is contrary to PDHG from the halophilic archaeon *Methanohalophilus mahii*, which prefers $NADP^+$ (Fischer et al. 1993). The PDHG activity of the *A. fulgidus* AroQ was not inhibited by prephenate or NAD^+ at concentrations up to 10 and 2 mM, respectively.

Chorismate mutase activity, which results in prephenate production, was inferred from the subsequent oxidation of prephenate and formation of NADH catalyzed by the PDHG activity of AroQ according to the following reactions:

$$\text{chorismate} \rightarrow \text{prephenate}$$

$$\text{prephenate} + \text{NAD}^{+} \rightarrow \text{hydroxyphenyl pyruvate} + \text{CO}_2 + \text{NADH}$$

In addition, CM activity was determined directly by measuring the decrease in chorismate absorbance at 274 nm. The CM specific activity estimated from this direct spectrophotometric assay was approximately 10 U/mg at 60°C, which is of the same order of magnitude as that of the associated PDHG activity. Due to instability of chorismate at higher temperature, a K_m value was not determined.

The PDT activity was determined qualitatively using LC/MS for detection of phenylpyruvate. To reduce background, the assay was conducted in water; inclusion of buffer compounds resulted in significantly increased noise levels. The mass spectrum showed a peak at 164 Da characteristic for phenylpyruvate that increased over the time of reaction (Fig. 1). Omission of either AroQ or prephenate from the reaction mixture resulted in no 164 Da peak. In addition, PDT activity was quantified spectrophotometrically by monitoring the appearance of phenylpyruvate at 320 nm in end-point assays. Using this assay, the specific activity was determined to be 0.51 U/mg at 77°C. The K_m for prephenate was estimated to be 1.3 ± 0.2 mM and the k_{cat}/K_m value was calculated to be 780 $M^{-1}s^{-1}$.

The calculated activation energy for AroQ PDHG was 60 kJ/mol over a 25–80°C temperature range (Fig. 2). This

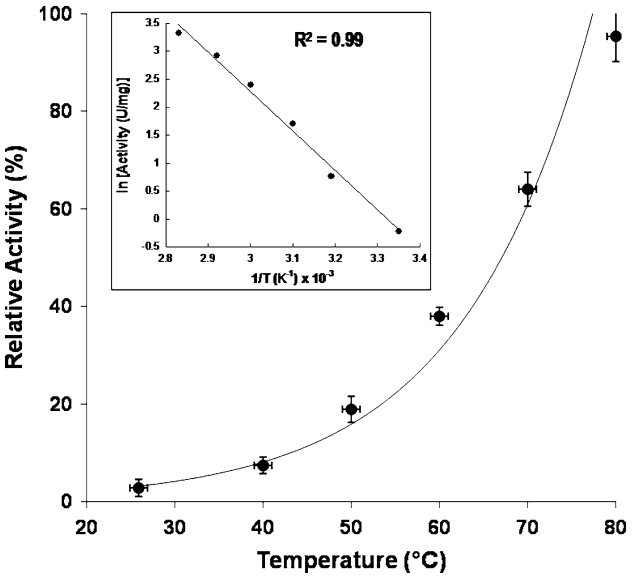


Fig. 2 Temperature dependency of *A. fulgidus* PDHG activity. Activity at 87°C corresponds to 50 U/mg. The Arrhenius plot (*inset*) was generated from temperature data over the range of 25–80°C

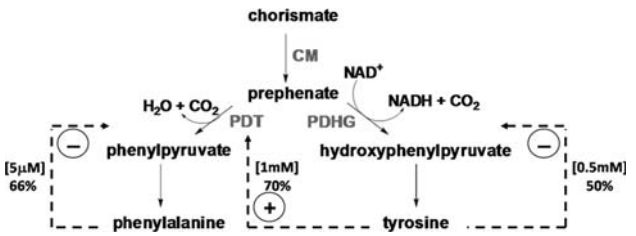


Fig. 3 End-product activation and inhibition of *A. fulgidus* PDHG/CM/PDT. *Dashed lines* represent activation (+) and inhibition (-) at the indicated ligand concentrations

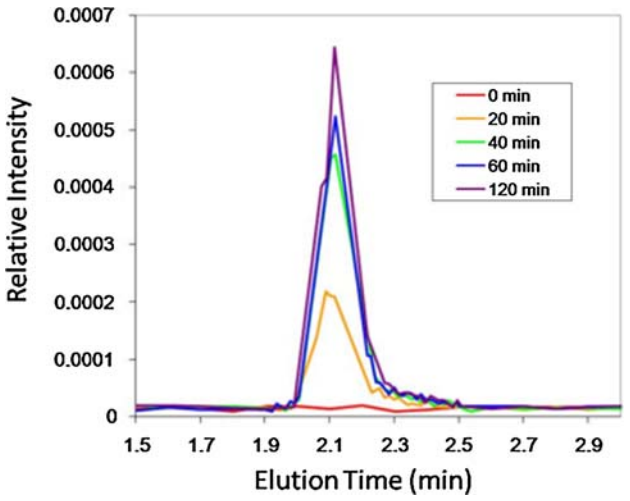


Fig. 1 The 164 *m/z* chromatogram of phenylpyruvate at the end of each PDT reaction period. The increase of 164-Da peak corresponds to the formation of phenylpyruvate

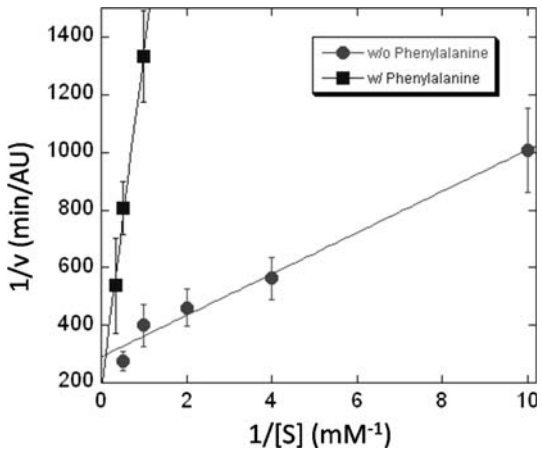


Fig. 4 Lineweaver-Burke plot of PDT in the absence and presence of 5 μM phenylalanine. The several magnitude increase of the slope in the presence of phenylalanine shows that phenylalanine acts as a competitive inhibitor to PDT

activation energy is similar to that determined for the PDHG from the hyperthermophilic bacterium *Aquifex aeolicus* (Bonvin et al. 2006). It has been suggested previously that high activation energy is a common feature of thermostable enzymes (D'Amico et al. 2003; Lim et al. 2004). The enzyme activity was maximal at 87°C and decreased by 40% at 95°C. The optimal temperature for PDHG activity was consistent with the growth temperature of *A. fulgidus*.

Effect of Phe and Tyr on enzyme activities

In bacteria, AroQ activities are often affected by Phe and Tyr, which control aromatic amino acid synthesis via feedback regulation. To examine product activation and inhibition of the *A. fulgidus* AroQ, CM, PDT, and PDHG activities were measured in the presence of Phe and Tyr (Fig. 3). CM activity was not affected by both amino acids at concentrations up to 0.5 mM. The lack of regulation of

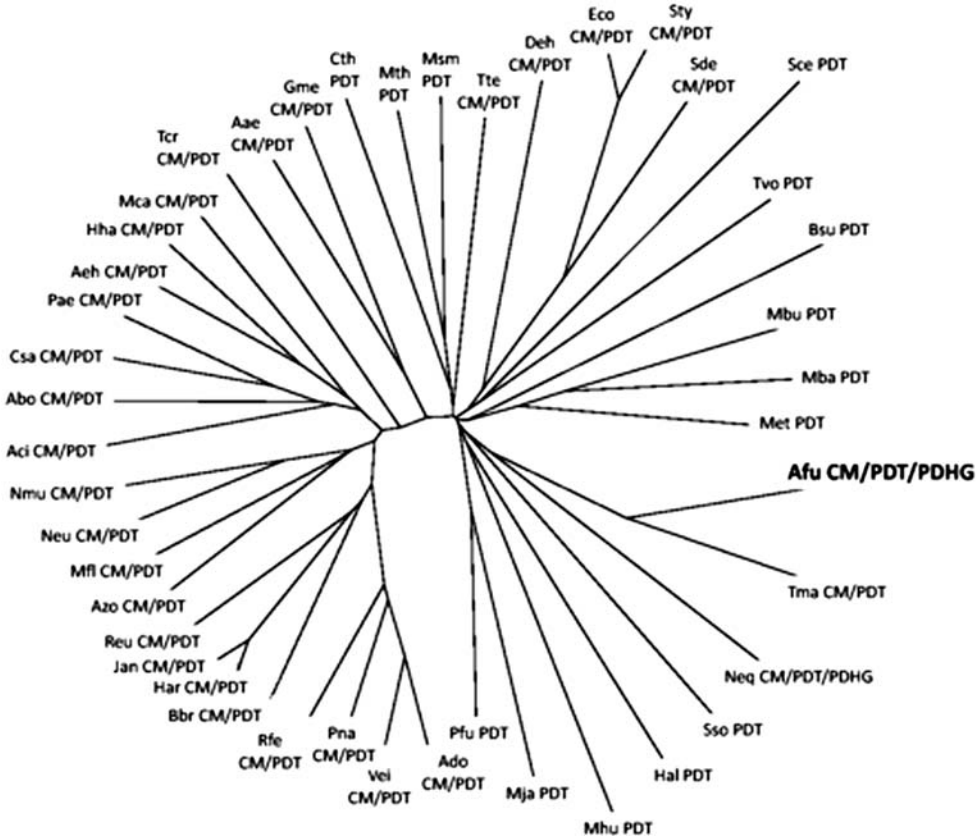


Fig. 5 Condensed phylogenic tree of selected prephenate dehydratases. The organisms, in alphabetical order with the accession number and organism abbreviations in parentheses are *Acidovorax* sp. strain JS42 (Ado, A1W8Q2), *Acinetobacter* sp. strain ADP1 (Aci, Q6FA94), *Alcanivorax borkumensis* strain SK2 (Abo, Q0VNQ1), *Alkalilimnicola ehrlichei* strain MLHE-1 (Aeh, Q0AA57), *Aquifex aeolicus* VF5 (Aae, O67085), *Archaeoglobus fulgidus* VC-16 (Afu, O30012), *Azoarcus* sp. strain EbN1 (Azo, Q5P7U8), *Bacillus subtilis* 168 (Bsu, P21203), *Bordetella bronchiseptica* RB50 (Bbr, Q7WGU3), *Chromohalobacter salexigens* strain DSM 3043 (Csa, Q1QVJ1), *Clostridium thermocellum* (Cth, A3DHN3), *Dehalococcoides* sp. strain CBDB1 (Deh, Q3ZZ17), *Escherichia coli* K-12 W3110 (Eco, P0A9J8), *Geobacter metallireducens* (Gme, Q39XC0), *Halobacterium* sp. NRC-1 (Hal, Q9HN73), *Halorhodospira halophila* strain DSM 244 (Hha, A1WUJ2), *Herminiimonas arsenicoxydans* (Har, A4G864), *Janthinobacterium* sp. (Jan, A6T1G6), *Methanobacterium thermoautotrophicum* (Mth, O27288), *Methanobrevibacter smithii* strain PS (Msm, A5UM29), *Methanococcoides burtonii* strain DSM 6242 (Mbu, Q12XR4), *Methanococcus jannaschii* (Mja, Q58054), *Methanosaeta thermophila* strain DSM 6194 (Met, A0B7Q1), *Methanosarcina barkeri* (Mba, Q46B73), *Methanospirillum hungatei* (Mhu, Q2FQ53), *Methylobacillus flagellatus* strain KT (Mfl, Q1H0N3), *Methylococcus capsulatus* (Mca, Q608S2), *Nanoarchaeum equitans* (Neq, Q74NC4), *Nitrosomonas eutropha* strain C71 (Neu, Q0AFR8), *Nitrosospira multiformis* strain ATCC 25196 (Nmu, Q2Y6Y7), *Polaromonas naphthalenivorans* strain CJ2 (Pna, A1VR17), *Pseudomonas aeruginosa* PA01 (Pae, Q9HZ67), *Pyrococcus furiosus* DSM 3638 (Pfu, Q8U408), *Ralstonia eutropha* strain JMP134 (Reu, Q46Y47), *Rhodoferax ferrireducens* strain DSM 15236 (Rfe, Q21Y52), *Saccharomyces cerevisiae* S288C (Sce, P32452), *Salmonella typhimurium* LT-2 (Sty, Q8ZMW7), *Shewanella denitrificans* strain OS217 (Sde, Q12KK0), *Sulfolobus solfataricus* (Sso, Q980P2), *Thermoanaerobacter tengcongensis* (Tte, Q8RB13), *Thermoplasma volcanium* (Tvo, Q97AD5), *Thermotoga maritima* (Tma, Q9WY02), *Thiomicrospira crunogena* strain XCL-2 (Tcr, Q31GD5), *Verminephrobacter eiseniae* strain EF01-2 (Vei, A1WMJ5)

CM activity by aromatic amino acids has also been observed in the methanogens, *M. mahii* and *Methanococcus maripaludis*, as well as *Mycobacterium tuberculosis* (Fischer et al. 1993; Porat et al. 2004; Kim et al. 2006). This is in contrast to the *E. coli* CM/PDT and CM/PDHG enzymes where the CM activity is feedback-inhibited by Phe and Tyr, respectively (Dosselaere and Vanderleyden 2001).

In general, bacterial and archaeal PDHG enzymes are regulated by end-product feedback inhibition (Koch et al. 1970, 1971; Friedrich et al. 1976; Porat et al. 2004; Bonvin et al. 2006). *A. fulgidus* AroQ PDHG activity is regulated similarly. The enzyme activity was inhibited by 50% in the presence of 0.5 mM Tyr, but insensitive to Phe at up to 5 mM. In contrast, AroQ PDT was activated by 1 mM Tyr resulting in a 70% increase in activity but was inhibited 66% by 5 μM Phe. The Phe inhibition of PDT was determined to be competitive with prephenate (Fig. 4). The overall inhibition and activation pattern of the *A. fulgidus* AroQ activities is similar to that observed for bacterial homologs (Friedrich et al. 1976), suggesting that this type of regulation is conserved across phylogenetic boundaries.

Phylogenetic analysis

The *A. fulgidus* AroQ amino acid sequence was used to identify homologs in the database. A representative collection of the more closely related homologs, all containing a PDT domain, was further assessed using phylogenetic analysis (Fig. 5). More distantly related are single-domain CM and PDHG enzymes, and these were not included in the phylogenetic tree.

The *A. fulgidus* AroQ is unusual in that it combines the CM, PDT, and PDH domains. Interestingly, the genome of *Nanoarchaeum equitans* suggests the presence of a closely related protein that also harbors the combined three domains (Fig. 5; Kleeb et al. 2006). Several proteins from extremophilic organisms cluster with the *A. fulgidus* AroQ. All except the CM/PDT enzyme from *Thermotoga maritima*, are of archaeal origin. Yanai et al. (2002) traced extensive horizontal gene transfer between *A. fulgidus* and *T. maritima*, which could explain the close relatedness between their proteins.

It is obvious that there is a great deal of flexibility exists in the arrangement of the three domains that catalyze the conversion of chorismate to phenylpyruvate and hydroxyphenylpyruvate within the archaea, bacteria and eukarya. This makes it questionable whether domain fusions have a selective advantage over single-domain enzymes. Based on the predominance of single or dual domain enzymes in all three kingdoms, we suggest that the trifunctional AroQ resulted from a gene fusion event. The hyperthermophilic archaeon, *N. equitans*, is thought to be a parasite of archaea such as *Ignicoccus hospitalis* and is known to have an extremely small and condensed genome (Waters et al. 2003). It is possible that gene fusion events are more likely to occur in condensed genomes. The evolutionary pressure leading to the formation of a trifunctional AroQ in *A. fulgidus* is less obvious. However, domain fusions may provide an alternative to supercomplexes for the efficient processing of labile substrates. The key intermediate, prephenate, is highly temperature labile. We speculate that CM/PDT and CM/PDH fusion proteins form complexes in other hyperthermophiles to convert efficiently this labile metabolite.

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