Extremophiles (2001) 5:229–239
Digital Object Identifier (DOI) 10.1007/s007920100201

ORIGINAL PAPER

Cristina Purcarea · Guy Hervé · Raymond Cunin
David R. Evans

Cloning, expression, and structure analysis of carbamate kinase-like carbamoyl phosphate synthetase from *Pyrococcus abyssi*

Received: December 8, 2000 / Accepted: March 8, 2001 / Published online: July 4, 2001

Abstract *Pyrococcus abyssi*, a hyperthermophilic archaeon found in the vicinity of deep-sea hydrothermal vents, grows optimally at temperatures around 100°C. Carbamoyl phosphate synthetase (CPSase) from this organism was cloned and sequenced. The active 34-kDa recombinant protein was overexpressed in *Escherichia coli* when the host cells were cotransformed with a plasmid encoding tRNA synthetases for low-frequency *Escherichia coli* codons. Sequence homology suggests that the tertiary structure of *P. abyssi* CPSase, resembling its counterpart in *Pyrococcus furiosus*, is closely related to the catabolic carbamate kinases and is very different from the larger mesophilic CPSases. *P. furiosus* CPSase and carbamate kinase form carbamoyl phosphate by phosphorylating carbamate produced spontaneously in solution from ammonia and bicarbonate. In contrast, *P. abyssi* CPSase has intrinsic bicarbonate-dependent ATPase activity, suggesting that the enzyme can catalyze the phosphorylation of the isosteric substrates carbamate and bicarbonate.

Key words Carbamate kinase · Carbamoyl phosphate synthetase · *Pyrococcus abyssi* · Evolution · Archaea · Hyperthermophily

Communicated by G. Antranikian

C. Purcarea · D.R. Evans (✉)
Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, MI 48201, USA
Tel. +1-313577-1016; Fax +1-313577-1510
e-mail: devans@cmb.biosci.wayne.edu

G. Hervé
Laboratoire de Biochimie des Signaux Régulateurs Cellulaires et Moléculaires, UMR CNRS 7631 – Université Pierre et Marie Curie, Paris, France

R. Cunin
Laboratorium voor Erfelijkheidsleer en Microbiologie, Instituut voor Moleculaire Biologie en Biotechnologie, Vrije Universiteit Brussel en Vlaams Interuniversitair Instituut voor Biotechnologie, Brussels, Belgium

Introduction

Hyperthermophilic organisms have evolved diverse adaptive mechanisms to the extreme temperatures of their environment. In addition to the obvious need to stabilize proteins and other potentially labile macromolecules, the extreme heat sensitivity of some small molecules generated in various metabolic pathways suggests that there may also be mechanisms for protecting unstable intermediates from thermal degradation. As an example, carbamoyl phosphate, although reasonably stable at 37°C, has a half-life of less than 2 s at 100°C (Allen and Jones 1964; Van de Casteele et al. 1990). Moreover, this intermediate decomposes to cyanate, a highly toxic substance.

Carbamoyl phosphate synthetases (CPSase; EC 6.3.5.5) from mesophilic bacteria are large heterodimeric enzymes (Meister 1989) that catalyze the synthesis of carbamoyl phosphate in a complex series of partial reactions (Anderson and Meister 1965, 1966; Powers and Meister 1978a, 1978b). Three reactions are involved in carbamoyl phosphate formation by the 120-kDa synthetase subunit of *Escherichia coli* CPSase:

$$\mathrm{ATP} + \mathrm{HCO_3^-} \rightarrow \text{carboxy phosphate} + \mathrm{ADP} \quad (1)$$

$$\text{Carboxy phosphate} + \mathrm{NH_3} \rightarrow \text{carbamate} + \mathrm{P_i} \quad (2)$$

$$\mathrm{ATP} + \text{carbamate} \rightarrow \text{carbamoyl phosphate} + \mathrm{ADP} \quad (3)$$

The intermediates in this series of reactions are unstable. Carboxy phosphate is hydrolyzed to CO_2 and inorganic phosphate with a half-life of milliseconds, whereas carbamate rapidly decomposes to ammonia and CO_2. In addition, most mesophilic CPSases have a second 40-kDa domain or subunit that catalyzes the production of ammonia from glutamine:

$$\text{Glutamine} + \mathrm{H_2O} \rightarrow \mathrm{NH_3} + \text{glutamate} \quad (4)$$

This reaction is required because ammonia, not ammonium ion, is the substrate for the synthetase subunit, and the concentration of the unionized species is low at physiological pH. The overall reaction is thus:

$$HCO_3^- + 2ATP + \text{glutamine} \rightarrow \text{carbamoyl phosphate} + 2ADP + \text{glutamate} + P_i \quad (5)$$

Although there are structural variants, the CPSase from nearly all known organisms has a molecular mass of 160 kDa and catalyzes the same series of partial reactions (Meister 1989). The only exception is the 160-kDa mammalian urea cycle enzyme CPSase I, which uses ammonia, not glutamine, as a nitrogen-donating substrate. Thus, it was surprising when Purcarea and collaborators (Purcarea et al. 1996) discovered that carbamoyl phosphate synthetase activity in *Pyrococcus abyssi* is associated with a 47-kDa protein that catalyzes the synthesis of carbamoyl phosphate from ATP, bicarbonate, and ammonia. *P. abyssi* is a strictly anaerobic sulfur-metabolizing hyperthermophilic and barophilic/barotolerant archaeon (Erauso et al. 1993) that grows optimally at 96°C. Similar results (Durbecq et al. 1997) were obtained for *P. furiosus* CPSase, and when the *P. furiosus* gene was cloned and sequenced, it was found to exhibit strong sequence similarity with carbamate kinases from mesophilic organisms (Uriarte et al. 1999).

Carbamate kinases (CKase; EC 2.7.2.2) are relatively small (31–37 kDa) homodimeric enzymes found in many prokaryotes (Mitruka and Costilow 1967; Abdelal et al. 1982; Cunin et al. 1986; Marina et al. 1994) that catalyze the formation of carbamoyl phosphate from ATP and carbamate formed chemically in solution from ammonia and bicarbonate (see Eq. 3). As one of the enzymes in the arginine deiminase pathway, carbamate kinases are thought to have a strictly catabolic role in metabolism, providing ATP from carbamoyl phosphate. However, the reaction that is catalyzed is reversible and, although the thermodynamics strongly favors the breakdown of carbamoyl phosphate, under appropriate conditions purified carbamate kinases have been shown to synthesize carbamoyl phosphate. A defining difference in mechanism between carbamate kinase and carbamoyl phosphate synthetases is that CPSases couple carbamoyl phosphate formation to the hydrolysis of a second ATP, thus allowing the reaction to proceed in the otherwise thermodynamically unfavorable direction.

Both the *P. abyssi* (Purcarea et al. 1996) and the *P. furiosus* (Durbecq et al. 1997) enzymes, similar to the mesophilic CPSases, were reported to use two ATP molecules for carbamoyl phosphate synthesis. The observation that the sequence closely resembles carbamate kinases, although the mechanism had certain features in common with the CPSases, led the authors to suggest that the archaeal enzymes may represent a "missing link" in the evolution of CPSases. However, Rubio and associates recently reported (Uriarte et al. 1999) that the catalytic mechanism of *P. furiosus* CPSase closely resembles that of carbamate kinase and involves the consumption of only one molecule of ATP.

In an attempt to resolve some of these discrepancies, the *P. abyssi* CPSase gene was cloned and expressed in both *Escherichia coli* and *Schizosaccharomyces pombe*. The primary structure of the enzyme was analyzed in comparison with those of the related carbamate kinases, CPSases, and other ATPases. Although *P. abyssi* CPSase is clearly closely related to carbamate kinases in structure, there are quantitative differences in the kinetic properties of this enzyme that suggest that it represents an intermediary form between CKases and CPSases.

Materials and methods

Cloning the *P. abyssi cpa* gene

The *cpa* gene, encoding CPSase, was cloned by polymerase chain reaction (PCR) amplification of *P. abyssi* chromosomal DNA. The oligonucleotides used in this study were

1. CK1: 5′-CAUCAUCAUCAUATGGGTAAGAGGGTAGTGATTGCACTTGGAGGTAACGC, TCT-3′
2. CK2: 5′-CUACUACUACUATTAAGGGAGAACTTGAGTACCAGTCTTCCCTTC-3′
3. RP1: 5′-GTACGTTCCCTTTTGACC-3′
4. RP2: 5′-GCTCACCTTGAGAAAGCT-3′
5. RP2C: 5′-AGCTTTCTCAAGGTGAGC-3′
6. BS: 5′-GGATCCGGAGAGAGTCGTCATAGCCCTCGG-3′
7. KE: 5′-GGTACCTCAGGGAATAACCTGTGTCCCGGTCTT-3′
8. YBS: 5′-TGGAATGGATCCGAGAGAGTCGTCATAGCC-3′
9. YBT: 5′-AAAAGGATCCTCAGGGAATAACCTGTGTCC-3′

P. abyssi chromosomal DNA was extracted from GE5 cells and purified using a CsCl gradient, as previously described by Charbonnier et al. (1992). PCR amplification was performed with a Perkin Elmer (Norwalk, CT, USA) Cetus DNA Thermal Cycler using the cycling parameters of 5 min denaturation at 94°C followed by 30 cycles of 94°C for 1 min, 46°C for 2 min, and 72°C for 2.5 min, with a final elongation step of 7 min at 72°C. The reaction mixture (50 µl) for amplification contained 200 ng of purified GE5 DNA template, 100 pmol of each of the 5′- and 3′-primers, CK1 and CK2, 200 µM deoxynucleoside triphosphate (dNTP) mix, and the buffer and units of Taq DNA polymerase specified by the manufacturer (Gibco/BRL, Bethesda, MD, USA). The amplified DNA fragment (1.3 kb) was cloned into the vector, pAMP1 (Gibco/BRL). The constructs were transformed into DH5α-competent cells (Gibco/BRL) and grown on plates containing Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin. Inverse PCR was then used to obtain the sequences at the 3′- and 5′-end of the gene. The template was prepared by *Bam*HI restriction of total GE5 DNA (1 µg) followed by religation with T4 DNA polymerase. The oligonucleotides RP1 and RP2 served as primers for PCR amplification.

For expression of the *P. abyssi* CPSase in *E. coli*, the *cpa* gene was amplified using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the 5′ BS and 3′ KE primers that contained *Bam*HI and *Kpn*I sites, respectively. The 1.3-kbp fragment was first inserted into the pCR Blunt vector (Invitrogen, Carlsbad, CA, USA) as described earlier. The insert was removed by cleaving with *Bam*HI and *Kpn*I and introduced into the corresponding sites of the

pRSETB expression vector (Invitrogen). The resulting plasmid, pCPSPA, was transformed into *E. coli* BL21 (DE3; Stratagene). The level of expression was optimized by prior transformation of the *E. coli* host cells with the plasmid pSJS1240 encoding AUA isoleucyl tRNA and AGA seryl tRNA (Kim et al. 1998). The doubly transfected cells were grown at 37°C for 18 h using LB medium supplemented by 100 μg/ml ampicillin and 50 μg/ml spectinomycin. A deletion mutant lacking 13 residues of the carboxyl end of *P. abyssi* CPSase was constructed as described earlier except that the primers YBS and RP2C, which incorporated *Bam*HI and *Pvu*II restriction sites, were used to amplify the truncated CPSase sequence. A codon for an additional glycine residue was introduced at the 3′-end of the construct as a consequence of cloning. The PCR product was subcloned between the *Bam*HI and *Pvu*II sites of pRSETA.

For expression in yeast, the *P. abyssi* CPSase gene was cloned into the yeast pESP1 vector and expressed in *Schizosaccharomyces pombe* (Stratagene). A DNA fragment encoding the CPSase gene was generated by PCR using *Pfu* DNA polymerase and the primers YBS and YBT, each containing a *Bam*HI site. The PCR product was then inserted into the vector pESP1 that had been cleaved with *Bam*HI. The resulting plasmid, pYCPS, was transformed into *S. pombe* for the expression of the *P. abyssi* enzyme as a fusion protein with the glutathione *S*-transferase (GST) polypeptide appended to the amino end of the recombinant CPSase.

Purification of the recombinant proteins

Pyrococcus abyssi CPSase and the deletion mutant expressed in *E. coli* could be purified in a single step by Ni^{2+} affinity chromatography. Cell extracts were fractionated on a ProBond column (Invitrogen). After elution with 200 mM NaCl to remove nonspecifically bound proteins, *P. abyssi* CPSase was eluted with 100 and 200 mM imidazol in 50 mM Tris-HCl, pH 8, and 200 mM NaCl. The protein expressed in yeast was also purified in one step by affinity chromatography of cell extracts on a GST affinity resin eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.

Enzyme assays

The ammonia-dependent CPSase activity was measured using a radiometric method (Robin et al. 1989). Carbamoyl phosphate formed from $[^{14}C]NaHCO_3$ was converted into carbamoyl aspartate in the coupled reaction with *E. coli* or *Aquifex aeolicus* aspartate transcarbamoylase. The activity was measured at 37°C or at 25°C when indicated, in a final volume of 0.3 ml, after incubation during 20 min, as previously described (Purcarea et al. 1996). The specific activity was expressed as micromoles per minute per milligram (μmol/min/mg) protein.

The rate of ADP formation in the presence or absence of ammonia was measured spectrophotometrically using pyruvate kinase/lactate dehydrogenase as coupling enzymes (Miran et al. 1991). The reaction mixture contained 120 mM NH_4Cl, 60 mM $NaHCO_3$, 0.7 mM MgATP, 50 mM Tris-HCl, pH 8, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 10 units of pyruvate kinase, and 20 units of lactate dehydrogenase in a total volume of 0.5 ml. Similar conditions were used to assay carbamate kinase. A similar method was used to measure the formation of ATP using carbamoyl phosphate and ADP as substrates (ATP synthetase activity) and a hexokinase/glucose-6-phosphate dehydrogenase coupling enzyme (Miran et al. 1991). The assay mixture contained 0.75 mM $NADP^+$, 0.2 mM ADP, 5 mM carbamoyl phosphate, 5 mM glucose, 1 mM $MgCl_2$, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase in a final volume of 0.5 ml.

Sequence analysis

Sequence comparison and multiple alignment were performed using the GAP and Pileup programs of the GCG software package (Needleman and Wunsch 1970). To search other genomes for open reading frames encoding the large CPSases, three query sequences corresponding to high conserved regions of mesophilic CPSases were employed:

1. EMNPRVSRSSALASKATGFPIAKVAAKLA
2. GPIVIGQACEFDYSGAQACKALR
3. GFPCIIRPSFTMGGSGGGIAYN

Modeling of the three-dimensional structure and energy minimization was performed using the program Swiss Model (Peitsch 1995, 1996; Guex and Peitsch 1997).

Results

Cloning and sequencing the *Pyrococcus abyssi cpa* gene

The *P. abyssi* gene encoding the CPSase was cloned by PCR amplification using purified total *P. abyssi* DNA as a template and primers complementary to the 5′- and 3′-end of the known *Pyrococcus furiosus cpa* sequence (Durbecq et al. 1997). The 1.3-kb PCR product was cloned into pAMP1 vector and the nucleotide sequence of the resulting clone (pPACK) was determined. Inverse PCR was then used to obtain the sequence of the extreme 5′- and 3′-ends of the *P. abyssi* gene and part of the upstream and downstream flanking regions. The PCR product was then cloned into the pCR blunt vector, yielding the recombinant plasmid pUDPACK. The combined sequence data obtained from pPACK and pUDPACK gave the entire sequence of the *P. abyssi* CPSase gene as well as 340 and 273 nucleotides of the 5′- and 3′-flanking regions, respectively (GenBank accession number AF081680). The gene has an open reading frame of 942 bp encoding a protein of 314 residues. The calculated molecular mass of the protein is 34,355 Da.

The deduced amino acid sequence is clearly homologous (Table 1) to *P. furiosus* CPSase, 87.9% identity, and the carbamate kinases of several different organisms, 42%–51% identity. In contrast, the *P. abyssi* enzyme sequence has only 13%–16% sequence identity with the CPSase from *E. coli*

Table 1. Sequence similarity[a] of *Pyrococcus abyssi* CPSase to carbamoyl phosphate synthetases, carbamate kinases (CK), and ATPases

Enzyme and organism	Identity (%)	Similarity (%)	Accession number
Archaea CK-like CPSases			
Pyrococcus horikoshii	93.6	98.4	AB009510
Pyrococcus furiosus	87.9	95.2	Y09829
Aeropyrum pernix	46.8	67.3	AP000063
Halobacterium salinarum	51.3	71.7	X80931
Eubacteria carbamate kinases			
Escherichia coli	42.8	66.3	U73857
Pseudomonas aeruginosa	50.7	70.3	X14693
Enterococcus faecium	47.4	70.8	AJ223332
Mycoplasma pneumoniae	42.3	67.7	AE000052 U00089
Clostridium perfringens	51.0	75.0	X97768
Haemophilus influenzae Rd	48.3	70.7	U32741 L42023
Synechocystis sp.	49.5	74.1	D90917 AB001339
CPSases and other ATPases			
Methanococcus jannaschii CarB1	14.2	45.8	U67577
Methanococcus jannaschii CarB2	16.0	42.3	U67578
Escherichia coli CarB	13.5	48.7	P00968
E. coli biotin carboxylase	16.9	50.0	P24182
E. coli glycine decarboxylase	17.8	49.2	P33195
E. coli GroEL	14.4	49.8	P06139

[a]Percent sequence identity and similarity percentages were obtained from the pairwise sequence alignment of the indicated protein with *P. abyssi* CPSase using the GAP program of the GCG 8.1 software package

and other mesophiles. This low level of sequence similarity is not significantly different from the 14%–16% identity with several unrelated ATP-binding enzymes such as biotin carboxylase, glycine decarboxylase, and the GroEL chaperonin from *E. coli*. The multiple alignment (Fig. 1) of the hyperthermophilic carbamate kinase-like CPSases and carbamate kinases further underscores the similarities of these two families of proteins. This comparison showed that 21% of the residues are conserved in all eight molecules and that the sequence similarity increases to 31% if conservative substitutions are included in the tally. There are four residues conserved only in thermophiles: Lys32, Trp88, Trp251, and Glu266. As observed for many hyperthermophilic enzymes, there are more charged residues and fewer thermal-sensitive residues than in the mesophilic enzymes. For example, compared to the mesophilic carbamate kinases, there are approximately three times as many lysines, twice as many histidines, and no cysteine residues in *P. abyssi* CPSase.

Expression and purification of *P. abyssi* CPSase

For expression in *E. coli*, the *P. abyssi* CPSase gene was amplified and subcloned into pRSETB, an expression vector that appends a 31-residue-chain segment containing six consecutive histidines to the amino end of the recombinant protein. One concern regarding heterologous expression of archaeal proteins in mesophilic bacteria is the disparate codon usage in these organisms. The scarcity of the correct tRNA molecules might be expected to limit the extent of expression. To partially compensate for the differences in eubacterial and archaeal codon usage, the *E. coli* host cells were pretransformed with the plasmid pSJS1240 encoding the AUA isoleucyl tRNA and the AGA seryl tRNA (Kim et al. 1998). These two codons are among the rarest in *E. coli* but are found in high frequency in the archaeal CPSase gene. The His-tagged recombinant protein was purified to near homogeneity (>95%) by chromatography on a Ni^{2+} affinity column (Fig. 2). A 1-l culture of doubly transformed *E. coli* yielded 62.1 mg of pure *P. abyssi* CPSase with a specific enzymatic activity of 0.40 ± 0.06 μmol/min/mg. It is notable that the yield of purified protein was reduced 3.5-fold in *E. coli* cells that were not transformed with pSJS1240.

P. abyssi CPSase directly isolated from GE5 cells exhibits high thermostability, with a half-life at 95°C of 3 h (Purcarea et al. 1996). The activity of the recombinant *P. abyssi* CPSase purified from *E. coli* was not affected as the temperature was increased in 5°C increments from 37° to 90°C (data not shown). Thus, the native and recombinant proteins have similar thermostability.

As an alternate approach, the *P. abyssi* CPS gene was cloned into the yeast pESP1 vector and expressed in *Schizosaccharomyces pombe*. This system generates a fusion protein that has GST appended to the amino end of the *P. abyssi* CPSase. The recombinant protein could be isolated in a single step by chromatography on a GST affinity column but had a specific enzymatic activity of only 0.014 ± 0.002 μmol/min/mg or 30-fold lower than the recombinant enzyme produced in *E. coli*. Cleavage of the GST fusion protein did not improve the activity, and consequently this approach was abandoned.

The extreme carboxyl end of the protein has been found (Marina et al. 1998) to be essential for catalytic activity of

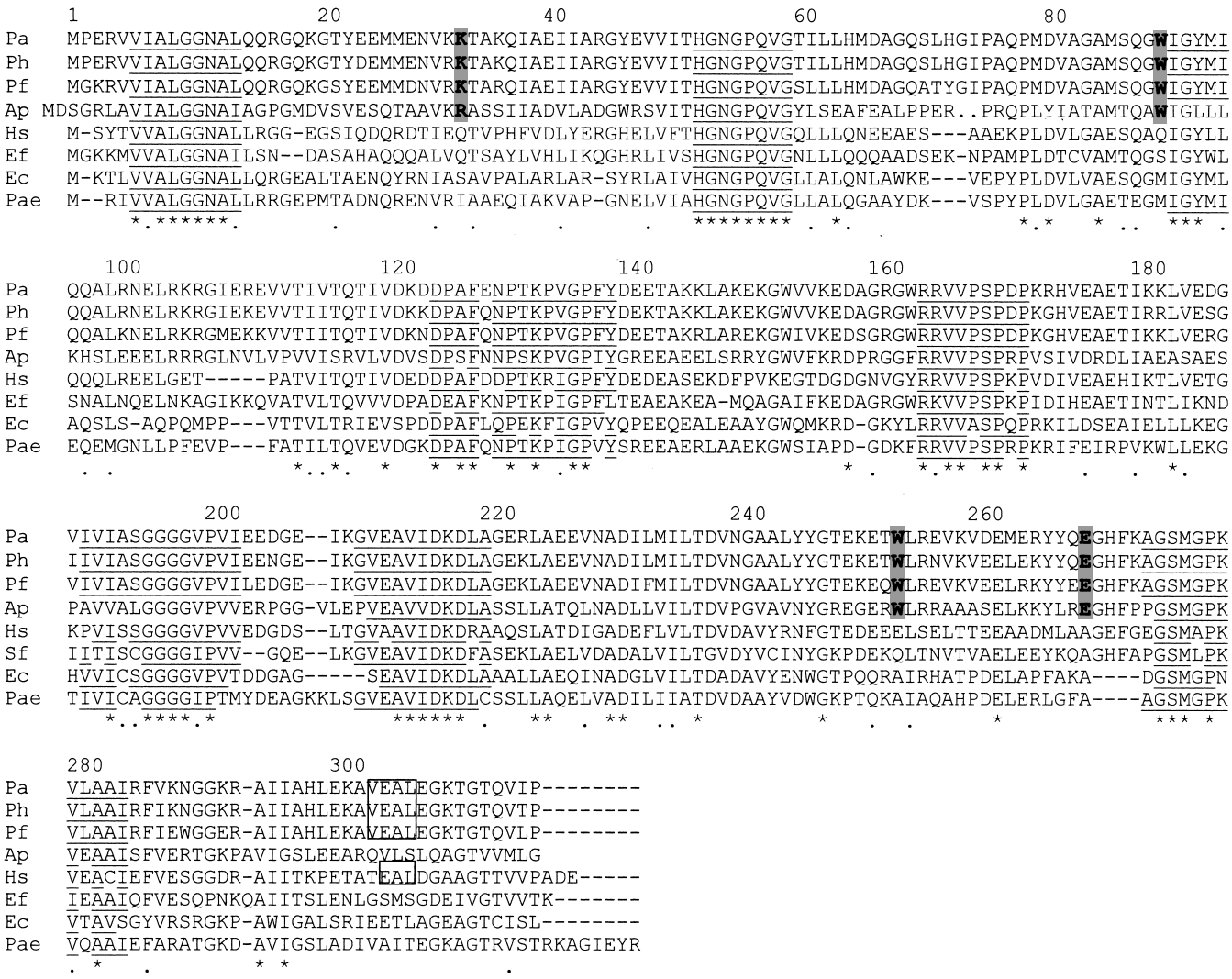

Fig. 1. Multiple sequence alignment of hyperthermophilic carbamate kinase-like (CK-like) carbamoyl phosphate synthetases (CPSases) and eubacterial CKases. Comparison of four hyperthermophilic CK-like CPSases: *Pyrococcus abyssi* (*Pa*); *Pyrococcus horikoshii* (*Ph*) (Kawarabayasi et al. 1998); *Pyrococcus furiosus* (*Pf*) (Durbecq et al. 1997); *Aeropyrum pernix* (*Ap*) (Kawarabayasi et al. 1999); and four mesophilic carbamate kinases: *Halobacterium salinarum* (*Hs*) (Ng et al. 2000); *Enterococcus faecium* (*Ef*) (Marina et al. 1998); *Escherichia coli* (*Ec*) (Blattner et al. 1997); *Pseudomonas aeruginosa* (*Pae*) (Stover et al. 2000). Conserved blocks (*underlined*) and identical (*stars*) or conserved (*dots*) residues occur in both hyperthermophilic and mesophilic proteins (*stars*); four residues are conserved only in the hyperthermophiles (*shaded*). The putative nucleotide-binding sequence at the carboxyl end of the protein, *VEAL*, is *boxed*

Enterococcus faecium carbamate kinase. To further explore the relationship between the archaeal CPSase and the eubacterial carbamate kinase, a deletion mutant was constructed lacking the last 13 residues of the carboxyl end of *P. abyssi* CPSase. The truncated protein was expressed in *Escherichia coli* and purified as described earlier for the parent molecule. The deletion mutant had a specific enzymatic activity of 0.12 µmol/min/mg, corresponding to a 70% decrease compared to the activity of the full-length protein. This result suggests that the carboxyl end of *P. abyssi* CPSase is important, but not essential, for catalysis. Moreover, the deletion mutant has thermostability similar to that of the full-length recombinant protein, an observation that indicates that the last 13 residues at the carboxyl end do not play a role in stabilizing the protein at elevated temperatures.

Kinetics of the recombinant *P. abyssi* CPSase

Previous studies (Purcarea et al. 1997) have established that *P. abyssi* CPSase is stable and maximally active at 90°C, near the optimal temperature for growth of this organism. However, for these studies we wished to compare the activity of *P. abyssi* CPSase and mesophilic carbamate kinase and CPSase, enzymes that are highly unstable at elevated temperature. Thus, the assays were conducted at lower temperatures. The Arrhenius plot obtained by measuring the activity of the *P. abyssi* enzyme at temperatures ranging from 35° to 95°C was linear and provided no indication of a temperature-dependent change in catalytic mechanism.

Mg^{2+}–ATP saturation curves (not shown) of the recombinant *P. abyssi* CPSase were measured in the presence and absence of 1 mM cytidine triphosphate (CTP), as it was pre-

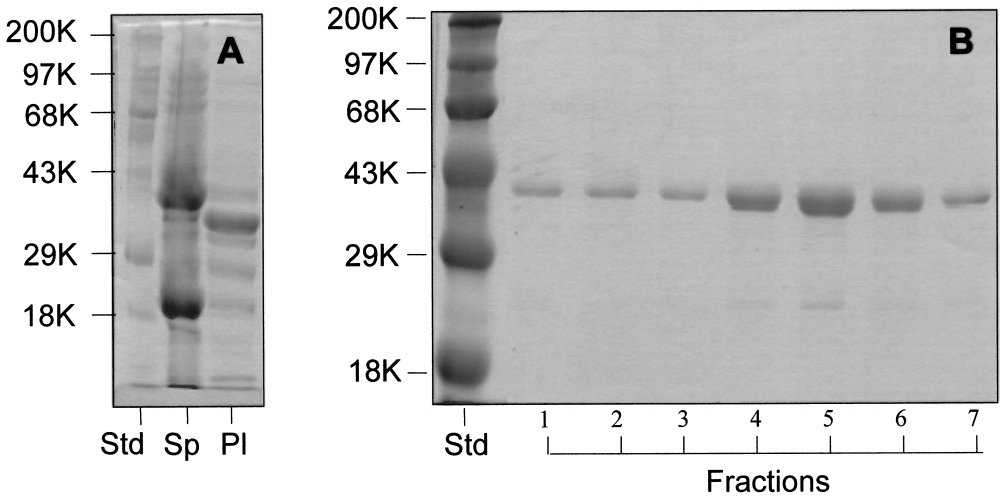

Fig. 2A,B. Purification of *P. abyssi* carbamoyl phosphate synthetase. A 100-ml culture of *Escherichia coli* cotransformed with pCPSA and pSJS1240 was grown overnight. The cells were suspended in 4 ml 50 mM Tris-HCl, pH 8, and lysed by sonication. Following centrifugation of the extract at 30,000 *g* for 15 min, the protein was found in the supernatant fraction (**A**, *Sp*) with only trace amounts in the pellet (**A**, *Pl*). The supernatant was applied to a 1.5-ml ProBond column (Invitrogen) equilibrated with 50 mM Tris-HCl, pH 8, and 200 mM NaCl. The column was washed with the same buffer to remove nonspecifically bound proteins. The *P. abyssi* CPSase was then eluted with 100 mM imidazol (**B**; *lanes [fractions] 1–3*) and 200 mM imidazol (**B**; *lanes [fractions] 4–7*) in the same buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluted fractions showed that the recombinant *P. abyssi* CPSase was nearly homogeneous and had a molecular mass of 38.5 kDa. The molecular mass of a series of standard proteins (*Std*, Sigma protein marker) is also shown

Table 2. Kinetic parameters obtained from ATP saturation curves[a]

Protein	CTP (mM)	Mg2+ excess (mM)	*V*max (μmol/min/mg)	*K*m ([S]0.5)b (μM)	*n*H
$CPSase_n$[c]	0	0	0.580 ± 0.003	84.0 ± 12.0	1.1 ± 0.1
$CPSase_r$	0	0	0.608 ± 0.027	59.7 ± 4.8	1
	1	0	0.278 ± 0.036	97.3[b]	1.4 ± 0.2
	1	2	0.240 ± 0.013	23.0 ± 5.6	1
CPSaseΔ[d]	0	0	0.271 ± 0.016	372 ± 45[b]	1.2 ± 0.1
	1	0	0.179 ± 0.012	303 ± 27[b]	2.1 ± 0.2
	1	2	0.099 ± 0.008	259 ± 60	1

CTP, cytidine triphosphate; $CPSase_n$, native CPSase; $CPSase_r$, recombinant CPSase

[a]The concentrations of sodium bicarbonate and ammonium chloride in the assay were held constant at 60 mM and 120 mM, respectively

[b]Nonlinear least squares fits of the data to the Michaelis–Menten equation and the Hill equations were obtained for all saturation curves; for those curves that exhibited detectable cooperativity, $[S]_{0.5}$ values are reported, whereas K_m values were obtained for the noncooperative saturation curves

[c]Results from Purcarea et al. (1996)

[d]A deletion mutant lacking the last 13 residues at the carboxyl end of *P. abyssi* CPSase

viously shown that this nucleotide partially inhibits the native enzyme (Purcarea et al. 1996). When the concentrations of $MgCl_2$ and ATP were equimolar, the saturation curve was hyperbolic and the V_{max} and K_m values (Table 2) were close to the values obtained for the enzyme isolated from *P. abyssi* cells (Purcarea et al. 1996), 0.5 μmol/min/mg and 84 μM, respectively. Sigmoidal saturation curves were obtained in the presence of 1 mM CTP. The Hill coefficient increased to 1.4, the K_m increased twofold, and the V_{max} was reduced by 40%. Again, similar results were reported for the enzyme directly purified from *P. abyssi* cell extracts. Thus, the wild-type and the recombinant protein exhibited similar kinetics. An excess of Mg^{2+} ion, which is required for the activity of *E. coli* and mammalian CPSase, had no effect in the absence of CTP. However, a 2 mM excess of Mg^{2+} in the reaction mixture containing 1 mM CTP completely abolished cooperativity without significantly altering the steady-state kinetic parameters.

The kinetic parameters of the deletion mutant were also measured. In the absence of CTP and the presence of excess Mg^{2+}, the ATP saturation curve was slightly sigmoidal (n^H = 1.2). The V_{max} was twofold lower than for the full-length enzyme, whereas the K_m increased sixfold. These altered kinetic parameters account for the moderate reduction in specific activity of the deletion mutant assayed at 0.75 mM ATP. CTP and excess Mg^{2+} affected the truncated protein (Table 2) in much the same way as the full-length protein. Thus, truncation of the carboxyl end of *P. abyssi* CPSase has little effect on the allosteric regulation of the protein. However, CTP inhibition of the

deletion mutant is somewhat less than observed with the full-length enzyme.

A comparison (Table 3) of the rate of bicarbonate-dependent ATP hydrolysis (see Eq. 1) and the rate of carbamoyl phosphate dependent ATP synthesis (the reverse of Eq. 3) for *P. abyssi* CPSase, *Enterococcus faecium* CKase, and *E. coli* CPSase reveals several interesting differences. The rate of ATP hydrolysis in the presence of ammonia, a measure of the overall rate of the reaction, was twice the rate of formation of carbamoyl phosphate (CPSase) formed for *P. abyssi* CPSase and *E. coli* CPSase, indicating a stoichiometry of two molecules of ATP consumed for the biosynthesis of one molecule of carbamoyl phosphate. In the case of carbamate kinase, the ratio of ATP consumed to carbamoyl phosphate formed is approximately 1 : 1.3.

Second, the rate of bicarbonate-dependent ATP hydrolysis by *P. abyssi* and *E. coli* CPSase is reduced 5-fold when ammonia is omitted from the assay mixture, yet hydrolysis still occurs at an appreciable rate. In contrast, the bicarbonate-dependent ATPase activity of carbamate kinase is barely detectable and is at least 50-fold lower in the absence of ammonia. Finally, the ATP hydrolysis catalyzed by *P. abyssi* and *E. coli* CPSase in the presence of ammonia occurs at approximately the same rate as carbamoyl phosphate-dependent ATP synthesis, whereas the hydrolytic reaction is 10-fold slower for *E. faecium* carbamate kinase. Although the tertiary folds of *E. faecium* carbamate kinase and *P. abyssi* CPSase are no doubt similar, the archaebacterial enzyme has kinetic characteristics that tend to favor carbamoyl phosphate synthesis.

Search of the *P. abyssi* genome for other CPSases

The kinetic studies suggest that the enzyme encoded by the *P. abyssi* CPSase has been adapted for carbamoyl phosphate synthesis. However, a recent report (Marina et al. 1999) suggested that, in addition to the small CK-like CPSase, the *P. furiosus* genome has two contiguous open reading frames that encode proteins homologous to the subunits of *E. coli* CPSase. The discovery of a second putative CPSase makes the role of the CK-like CPSase in *P. furiosus* metabolism unclear.

Consequently, three query sequences corresponding to highly conserved region in the large mesophilic CPSases (see Materials and methods) were employed to search the genomes of *P. abyssi* and two other hyperthermophiles to determine whether these organisms also encode a second putative CPSase. Using the search algorithms described in Materials and methods, all three query sequences located the *car*A and *car*B open reading frames previously identified in the 1.8 Mb *P. furiosus* genome. However, no search with any of the query sequences revealed an open reading frame or region of significant homology to the mesophilic CPSases in the *P. abyssi*, *Pyrococcus horikoshii*, or *A. pernix* genome. Thus, in these organisms, the only known protein that could potentially synthesize carbamoyl phosphate is the small CK-like CPSase described in this article.

Modeling of the *P. abyssi* CPSase

The strong sequence homology between *P. abyssi* CPSase, *E. faecium* carbamate kinase, and the *P. furiosus* enzyme suggests that all these proteins have the same fundamental tertiary structure. However, there are significant differences in the kinetics between *P. abyssi* CPSase and both these other proteins. Consequently, we modeled the tertiary structure of the *P. abyssi* enzyme using both *E. faecium* CKase (Marina et al. 1999) and *P. furiosus* CPSase (Ramon-Maiques et al. 2000) as a template. Both template proteins gave plausible model structures; however, not surprisingly in view of the strong sequence similarity, the backbones of the *P. abyssi* and *P. furiosus* enzymes (Fig. 3) are virtually superimposable.

The ATP-binding region of the *P. furiosus* CPSase active site has been identified because the crystals were found to contain bound ADP (Fig. 3) that carried through the isolation and crystallization procedure. A representation of the side chains of residues in contact with ADP (shown in red in Fig. 3) shows that the nucleotide is intimately cradled within a narrow cleft. With one notable exception described here,

Table 3. Reactions catalyzed by *Pyrococcus abyssi* CPSase, *Enterococcus faecium* CKase, and *Escherichia coli* CPSase

Enzyme	Activity (μmol/min/mg)[a]			
	CPSase[b]	HCO_3^--dependent[b] ATPase, with NH_3	HCO_3^--dependent ATPase, no NH_3	CP-dependent ATP synthetase
P. abyssi CPSase	0.075 ± 0.018	0.153 ± 0.043	0.023 ± 0.003	0.032 ± 0.003
E. faecium Ckase	0.317	0.427	0.008	4.533
E. coli CPSase[c]	1.00	2.00 ± 0.40	0.40 ± 0.05	0.39 ± 0.05

[a] The bicarbonate-dependent CPSase reaction was measured in the presence (with NH_3) and absence (no NH_3) of 120 mM ammonium chloride. The CPSase, ATPase, and carbamoyl phosphate-dependent (CP-dependent) ATP synthetase assays were conducted at 24°C and pH 7.4, as described in Materials and methods

[b] Both reactions assay the overall synthesis of carbamoyl phosphate under the same conditions but measure different products. In the CPSase assay, carbamoyl phosphate production is measured whereas the HCO_3^--dependent ATPase assay measures the production of ADP

[c] Results from Miran et al. (1991)

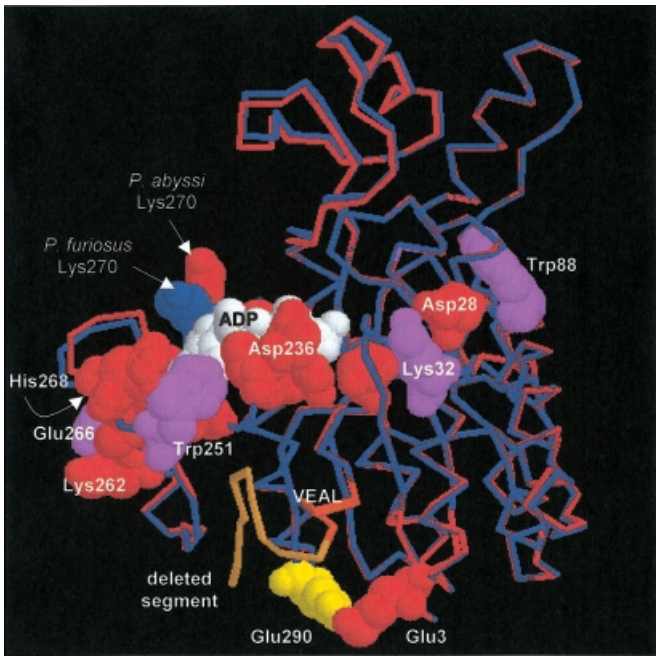

Fig. 3. Model of *P. abyssi* CPSase. The tertiary structure of *P. abyssi* CPSase was modeled using the structure of *P. furiosus* CPSase (Ramon-Maiques et al. 2000) as the template. Only one monomer of the dimeric protein is shown in the diagram. The dimer interface corresponds to the *right side* of the molecule as depicted in the diagram. The backbone of the *P. abyssi* enzyme (*red*) is virtually superimposable on the backbone of *P. furiosus* CPSase (*blue*). The residues that form the ADP-binding site are *space filled*. The residues that are conserved in all the hyperthermophilic enzymes but not in the mesophilic enzymes are shown in space-filling representation (*violet*). The segment deleted from the carboxyl end of the polypeptide (*orange*) that includes the putative nucleotide-binding consensus sequence is shown in *red-orange*

the side chains of the active site residues of *P. abyssi* and *P. furiosus* CPSase have the same orientation. However, there are significant differences in the active sites of the archaeal CK-like CPSase and the mesophilic carbamate kinase. Gly236 in *E. faecium* carbamate kinase is replaced with aspartate in *P. abyssi* CPSase. One of the Asp236 δ-carboxyl oxygens is located 2.6 Å from an α-phosphate oxygen of ADP. Ala270 in *E. faecium* carbamate kinase is replaced by a lysine in *P. abyssi*. In *P. furiosus* CPSase, the Lys270 side chain extends away from the ADP-binding site, whereas in *E. faecium* CPSase this side chain shifts into position over the α-phosphate of the bound ADP.

Although this variation may represent a real difference in the *P. abyssi* and *P. furiosus* CPSase, it is more likely to reflect a conformational change induced by nucleotide binding. The X-ray structure of the *P. furiosus* CPSase was solved as a complex with ADP, whereas the energy minimization of the *P. abyssi* CPSase model was carried out without the bound nucleotide. In *P. abyssi* CPSase, the carbamate kinase residue Gln251 is replaced with tryptophan, the side chain of which lies in close proximity to the adenine ring. Trp251 is one of only four residues (shown in violet in Fig. 3) conserved in all the thermophilic enzymes but not in the mesophilic carbamate kinases. One of these residues, Glu266, participates in two salt links with Lys262 and with His268. The carbonyl oxygen of His268 is located within hydrogen-bonding distance of the N-6 atom of the bound adenine. All these differences would be expected to increase the affinity of the enzyme for the nucleotide in the hyperthermophilic enzymes and may be associated with the observed differences (Table 3) in the kinetics of *P. abyssi* CPSase and *E. faecium* carbamate kinase. The other conserved residues (Fig. 3, violet) are Trp88, part of a hydrophobic patch at the subunit interface, and Lys32, which forms a salt link with Asp28.

Discussion

In *Pyrococcus abyssi*, carbamoyl phosphate is synthesized from ATP, bicarbonate, and NH_3 in a reaction catalyzed by an unusually small 34-kDa protein (Purcarea et al. 1996). This observation was unprecedented, because in all mesophilic organisms the synthesis of carbamoyl phosphate in the de novo pyrimidine biosynthesis is catalyzed by a 160-kDa protein that uses glutamine as the nitrogen-donating substrate. We have now cloned the *P. abyssi* CPSase, determined its sequence, and expressed the protein in *E. coli* and yeast.

As shown for the homologous enzymes from *P. furiosus* (Durbecq et al. 1997), *P. horikoshii* (Kawarabayasi et al. 1998), *A. pernix* (Kawarabayasi et al. 1999), and of the halophilic archaeon *Halobacterium salinarum* (Ruepp and Soppa 1996), the sequence of *P. abyssi* CPSase is clearly homologous to the *arcC* genes coding for carbamate kinase in mesophilic bacteria. For example, there is a 48%–51% sequence similarity between the *P. abyssi* enzyme and the carbamate kinases, indicative of a similar tertiary fold. In contrast, only minimal sequence similarity, 13.5% identity, exists between *P. abyssi* CPSase and *E. coli* CPSase sequence. Therefore, these small archaebacterial CPSases have been designated CK-like CPSases. Similar modeling studies (unpublished) suggested that *P. abyssi* CPSase has the same tertiary fold as *E. faecium* carbamate kinase (Marina et al. 1999). Recently, Ramon-Maiques and coworkers (2000) have solved the structure of the homologous *P. furiosus* CK-like CPSase to a resolution of 1.5 Å. This structure clearly established that this CK-like CPSase has the same tertiary fold as *E. faecium* carbamate kinase, one that is distinctly different from the large mesophilic CPSases as typified by the *E. coli* enzyme. Thus, from a structural standpoint, the CPSase from *P. abyssi* and several other Archaea are carbamate kinases.

An analysis of the amino acid composition of the *P. abyssi* enzyme and other CK-like CPSases showed that, as observed in other thermophilic proteins, there are more ionic residues, such as lysine and histidine, and fewer thermolabile residues such as cysteine compared to their mesophilic counterparts. The availability of the *P. furiosus* CPSase X-ray structure and the sequences of four hyperthermophilic CK-like CPSases allow a more detailed analysis. Rubio and associates (Ramon-Maiques et al. 2000) postulated that thermostability could be attributed to (1) the more hydrophobic interface that stabilizes the dimer

and (2) a large number of surface salt links which stabilize the tertiary structure of the monomer. The dimer interface, a highly conserved region in all CK-like CPSases, includes Trp88, part of the hydrophobic patch found only in the hyperthermophilic proteins. Moreover, there are 14 pairs of oppositely charged residues on the surface of the *P. abyssi* structure that have their charges properly oriented and within 2.8 and 3.6 Å. All these salt links are conserved in *P. abyssi* and *P. furiosus*, with one exception that underscores the importance of these interactions: Lys3, a residue that forms a salt link with Glu290 in *P. furiosus*, is replaced with a residue of opposite charge, a glutamate, in *P. abyssi* (see Fig. 3). This salt link may be especially important for thermostability because it ties together the amino and carboxyl segments of the polypeptide.

Recently, an open reading frame was discovered in the *P. furiosus* genome that is homologous to the larger mesophilic CPSases. The possibility exists that in this organism the carbamoyl phosphate synthesis is catalyzed by this large, as yet uncharacterized, CPSase homologue and that the small CK-like CPSase is involved in arginine degradation or has other functions unrelated to pyrimidine biosynthesis. An alignment of the sequences of the putative *P. furiosus* large CPSase and *E. coli* CPSase shows that 22 residues of the 25 residues implicated in CPSase activity and substrate binding (Thoden et al. 1997) are present in this putative CPSase, suggesting that this *P. furiosus* CPSase homologue may be functional. Using a search strategy that rapidly revealed the presence of the putative larger CPSase in the *P. furiosus* genome, we were unable to find a homologous protein in the *P. abyssi, P. horikoshii*, or *A. pernix* genomes.

However, all these organisms possess a CK-like CPSase. Thus, while the situation in *P. furiosus* remains to be clarified, it appears that in several other Archaea the synthesis of carbamoyl phosphate is mediated solely by a CK-like CPSase. This interpretation is consistent with the failure to detect any other glutamine- or ammonia-dependent CPSase activity in *P. abyssi* cells (Purcarea et al. 1996). In this regard, it is very interesting to note that recent studies (Alcantara et al. 2000) showed that a plasmid encoding *P. furiosus* CKase can complement an *E. coli* mutant lacking the endogenous CPSase.

Further support for the involvement of *P. abyssi* CPSase in de novo pyrimidine biosynthesis come from the observation that, unlike the homologous *P. furiosus* enzyme or the mesophilic carbamate kinases, the *P. abyssi* enzyme is allosterically regulated by pyrimidine nucleotides (Purcarea et al. 1996). The location of the nucleotide-binding site has not been identified. However, the *P. abyssi* enzyme contains the consensus sequence VEAL (see Fig. 1) found in the allosteric site of *E. coli* ATCase and other proteins regulated by nucleotides (Traut 1994). Consequently, 13 residues, including this signature sequence (Fig. 3), were deleted from the carboxyl end of the *P. abyssi* enzyme. Although the catalytic activity of the deletion mutant was reduced by 70%, the residual activity was still inhibited by CTP (Table 2), although not as effectively as the native enzyme. This result indicates that the carboxyl end of the polypeptide and the VEAL consensus sequence are not essential but may be involved in the allosteric regulation of this enzyme.

Although both CPSases and carbamate kinases can synthesize carbamoyl phosphate, the overall strategy for the biosynthetic reaction is quite different. Carbamate kinases directly phosphorylate carbamate formed chemically in solution from NH_3 and bicarbonate, but the CPSase mechanism is more complex and involves the concerted action of two different catalytic sites. At one site, CPS.A, ATP phosphorylates bicarbonate to form carboxy phosphate, which then reacts spontaneously with ammonia, usually derived from glutamine hydrolysis, to form carbamate. Carbamate is thought to diffuse through an intramolecular tunnel to the second site, CPS.B, where it is phosphorylated by a second ATP molecule. Thus, the major distinction is that CPSases can catalyze the formation of carbamate, whereas carbamate kinases catalyze only the second partial reaction that occurs on CPS.B.

The coupling of the hydrolysis of a second ATP molecule makes the formation of carbamoyl phosphate by the CPSases irreversible and the biosynthetic reaction thermodynamically favorable. Carbamate kinases cannot form carbamoyl phosphate via this more complex route because (1) these enzymes lack bicarbonate-dependent ATP hydrolytic activity and therefore cannot form carboxy phosphate, and, (2) although carbamate kinases are dimeric, there is no tunnel connecting the catalytic sites that would allow passage of carbamate. Thus, the formation of carbamoyl phosphate occurs at a single site. The presumed structural similarity of *P. abyssi* CPSase with carbamate kinases makes it very likely that the catalytic mechanism is similar in both enzymes.

Despite the differences in structural organization, the phosphorylation reactions catalyzed by CPSase, and carbamate kinases may proceed through a common mechanism. The two major functional domains of CPSase, CPS.A and CPS.B, catalyze very similar reactions, the phosphorylation of the isosteric substrates bicarbonate and carbamate. The two domains have been shown in the CPSases of mammals, *E. coli* (Guy and Evans 1996), and yeast (Serre et al. 1999) to be functionally equivalent in the sense that both CPS.A and CPS.B catalyze both partial reactions. Although the details of the carbamate kinase mechanism remain to be learned, it is reasonable to expect that carbamate phosphorylation, identical to the reaction catalyzed by CPS.B, occurs via the same or very similar mechanism as that operative in CPSases.

The mechanism of the CK-like CPSases has been controversial. Measurements of stoichiometry of substrate consumption and product formation for the reaction catalyzed by *P. abyssi* CPSase showed that 2 moles of ATP were hydrolyzed for each mole of carbamoyl phosphate synthesized. Identical results were obtained for the *P. furiosus* enzyme (Durbecq et al. 1997). These studies suggested that carbamoyl phosphate formation proceeded through the classical mechanism in which the enzyme catalyzes carbamate formation in situ. More recently, Rubio and his associates (Uriarte et al. 1999) carefully reexamined this issue using recombinant *P. furiosus* CPSase and obtained very different results. These authors found that the formation of 1 mole of carbamoyl phosphate is accompanied by the hydrolysis of only 1 mole of ATP and the release of 1 mole

of inorganic phosphate, a result consistent with a carbamate kinase-like mechanism. The authors suggested that the discrepancy could be accounted for by the presence of contaminating ATPase in the protein isolated from *P. abyssi* and *P. furiosus* cell extracts.

The results reported here tend to support the previous studies of *P. abyssi* CPSase, in that (1) in contrast to the *P. furiosus* enzyme, *P. abyssi* CPSase clearly catalyzes bicarbonate-dependent ATP hydrolysis in the absence of NH_3; (2) in the presence of NH_3, the ATPase activity increases appreciably; (3) carbamoyl phosphate synthesis is accompanied by the hydrolysis of 2 moles of ATP; and (4) the reaction rate of the ATP synthesis is lower than that of the ATP hydrolysis. The enzyme isolated from *P. abyssi* cells was purified to homogeneity and the recombinant protein, which has nearly identical kinetic properties, is at least 95% pure. Therefore, it is unlikely that a contaminating bicarbonate-dependent ATPase is responsible for these observations.

These results suggest that the intrinsic bicarbonate-dependent ATPase activity represents the critical difference between *P. abyssi* CPSase and either *P. furiosus* CPSase or the carbamate kinases, enzymes that lack this activity (Uriarte et al. 1999) and, therefore, cannot catalyze the formation of carboxy phosphate (Eq. 1). The ability of the *P. abyssi* enzyme to bind and phosphorylate bicarbonate, as well as carbamate, could be brought about by relatively subtle changes in the active site given the similarity of these two potential substrates.

The observation that the enzyme binds and phosphorylates both carbamate and bicarbonate may account for the difference observed in the stoichiometry of the reaction. One ATP would be utilized in the formation of carbamoyl phosphate from carbamate in equilibrium with bicarbonate and ammonia in solution. On the other hand, the binding and phosphorylation of bicarbonate consume a second mole of ATP in the formation of carboxy phosphate. The intermediate would either be released from the enzyme and hydrolyzed, equivalent to an intrinsic, albeit wasteful, ATPase side reaction or react with ammonia to form carbamate. If the carbamate is sequestered, it may be phosphorylated by a second ATP molecule leading to the formation of carbamoyl phosphate. Depending on the fate of the carboxy phosphate formed when bicarbonate binds to the enzyme, the stoichiometry would lie somewhere between 1 and 2 moles of ATP per mole of carbamoyl phosphate formed, and the ratio may critically depend on the assay conditions. This mechanism would also account for the reversibility of the reaction observed for the *P. furiosus* enzyme (Uriarte et al. 1999) and the *P. abyssi* enzyme (unpublished results). Further kinetic studies designed to assess the merit of this proposal are in progress.

What then ensures that the reaction catalyzed by *P. abyssi* CPSase proceeds in the thermodynamically unfavorable direction toward carbamoyl phosphate synthesis? A possible explanation is that the carbamoyl phosphate once formed is channeled directly to the active site of aspartate transcarbamoylase, the next enzyme in the pyrimidine biosynthetic pathway. Channeling would not only prevent the enzymatic hydrolysis of newly formed carbamoyl phosphate but would also provide protection for this highly labile intermediate from thermal degradation. Such a carbamoyl phosphate channeling has been documented in *P. abyssi* CPSase (Purcarea et al. 1999), suggesting that the enzyme forms a complex with ATCase and with OTCase, a homologous enzyme that initiates arginine biosynthesis.

In summary, *P. abyssi* CPSase has been cloned and expressed at high levels in *E. coli.* The purified recombinant protein has thermostability and catalytic and regulatory parameters comparable to those of the native enzyme isolated from *P. abyssi* cell extracts. The *P. abyssi* enzyme, like *P. furiosus* CPSase, is closely related in structure to the carbamate kinases, but differs in that it possesses an intrinsic bicarbonate-dependent ATPase activity.

Acknowledgments We thank Dr. Vincente Rubio for helpful discussions of his results with the *Pyrococcus furiosus* CPSase before publication and Dr. Steve Sandler for his generous gift of the plasmid pSJS1240. This research was supported by a grant (MCB-9810325) from the National Science Foundation.

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