The hyperthermophiles *Pyrococcus furiosus* and *Pyrococcus abyssi* make pyrimidines and arginine from carbamoyl phosphate (CP) synthesized by an enzyme that differs from other carbamoyl-phosphate synthetases and that resembles carbamate kinase (CK) in polypeptide mass, amino acid sequence, and oligomeric organization. This enzyme was reported to use ammonia, bicarbonate, and two ATP molecules as carbamoyl-phosphate synthetases to make CP and to exhibit bicarbonate-dependent ATPase activity. We have reexamined these findings using the enzyme of *P. furiosus* expressed in *Escherichia coli* from the corresponding gene cloned in a plasmid. We show that the enzyme uses chemically made carbamate rather than ammonia and bicarbonate and catalyzes a reaction with the stoichiometry and equilibrium that are typical for CK. Furthermore, the enzyme catalyzes actively full reversion of the CK reaction and exhibits little bicarbonate-dependent ATPase activity. In addition, it cross-reacts with antibodies raised against CK from *Enterococcus faecium* and its three-dimensional structure, judged by x-ray crystallography of enzyme crystals, is very similar to that of CK. Thus, the enzyme is, in all respects other than its function in vivo, a CK. Because in other organisms the function of CK is to make ATP from ADP and CP derived from arginine catabolism, this is the first example of using CK for making rather than using CP. The reasons for this use and the adaptation of the enzyme to this new function are discussed.

In this reaction one ATP molecule is used/molecule synthesized of CP, and the true substrate that is phosphorylated is carbamate, which is generated chemically from bicarbonate and ammonia (1–3). Because of the unfavorable equilibrium of the reaction, CK is thought to function in vivo exclusively in the direction of ATP synthesis using the CP generated by catabolic ornithine transcarbamylase in the fermentative catabolism of arginine (4).

In contrast to CK, CPS synthesizes irreversibly the CP that is used in the biosynthesis of pyrimidines, arginine, and urea, according to the following reaction (5).

$$2\text{ATP} + \text{HCO}_3^- + \text{NH}_3 (\text{glutamine}) \rightarrow 2\text{ADP} + \text{Pi} + \text{CP} (+\text{glutamate})$$

(Eq. 2)

The reaction catalyzed by CPS differs from that catalyzed by CK not only in its irreversibility in the direction of CP synthesis, but also in the use of bicarbonate and ammonia as the true substrates and in the utilization of two ATP molecules/molecule made of CP.

CK and CPS also differ structurally. CK is a homodimer of a polypeptide of approximately 33 kDa (6), whereas CPS is a 120-kDa polypeptide that is either associated or fused to another polypeptide of approximately 40 kDa (7). Alignment of the amino acid sequences of CK and CPS failed to reveal the existence of a statistically significant sequence identity between the two enzymes (8), whereas there is a high degree of sequence identity among different CKs (6) or different CPSs (9). No obvious structural similarities are found when the recently determined three-dimensional structures of CPS from *Escherichia coli* (10) and of CK from *Enterococcus faecalis* (11) are compared. The two proteins exhibit an open β-sheet αβ structure. Whereas CPS exhibits the fold found in biotin carboxylase and in other proteins that synthesize acylphosphate bonds, the CK fold appears not to be represented in structural data bases, although it is likely to be found in other enzymes of presently unknown structure that synthesize acylphosphates and exhibit sequence similarity with CK, such as acetylglutamate kinase, γ-glutamyl kinase, and long chain fatty acyl-CoA synthetases (6).

Given the important differences between CPS and CK, the recent description of CK-like CPSs in the hyperthermophilic archea *Pyrococcus abyssi* (12) and *Pyrococcus furiosus* (13, 14) is puzzling. In the extracts of these extremophiles that live at 100 °C and, in the case of *P. abyssi*, at high pressure in the ocean bottom, the CK-like CPS was the only activity found to synthesize CP in reaction mixtures containing ATP, bicarbonate, and ammonia (12–14). The polypeptide mass, homodimeric nature, and amino acid sequence (reported only for *P. furiosus*) of these pyrococcal enzymes (12, 14) are characteristic of CKs. However, similarly to CPSs, these enzymes were reported (12,
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14) to use two ATP molecules/molecule made of CP and to exhibit ATPase activity in the absence of ammonia, although the magnitude of the ATPase was greater, relative to the overall reaction, than in classical CPSs (15, 16). In contrast with most CPSs, which use glutamine with preference to ammonia as the nitrogen source (7), the pyrococcal CPSs use exclusively ammonia (12–14) but this is also the case with the ureotelic CPSs (7). Given these puzzling characteristics of pyrococcal CPS, we decided to study it in detail as it might represent an intermediate step in the evolution of CP biosynthesis (14).

Thus, we have cloned and hyperexpressed in _E. coli_ the gene encoding the CPS from _P. furiosus_, and we have purified and crystallized the recombinant enzyme generated in _E. coli_. The large amounts of pure protein obtained in this way have permitted us to study the stoichiometry, reversibility, point of equilibrium, and the nature of the substrates in the reaction. Our results unequivocally show that the enzyme catalyzes the CK reaction. Furthermore, our initial results of x-ray studies on enzyme crystals also indicate that the structure of this enzyme resembles closely that of CK. Therefore, this appears to be the first example of a CK with an anabolic role that is reserved in other organisms for CPS (the synthesis of CP as a precursor of arginine and the pyrimidines).

### EXPERIMENTAL PROCEDURES

**Materials**—Recombinant enterococcal CK was isolated from _E. coli_ BL21 (DE3) cells (obtained from Novagen) transformed with the plasmid pCK41 exactly as described (6). The preparation and characterization of monoclonal antibodies mAbCK1, mAbCK2, which recognizes epitopes within the C-terminal 14 residues of enterococcal CK, and mAbCK3, which recognizes an epitope toward the center of the polypeptide, have been reported already (6). Polyclonal monospecific antisera against enterococcal CK were prepared by immunization of rabbits with the purified recombinant enzyme following a standard immunization protocol (17). _E. coli_ CPS was purified as described (18). Pyruvate kinase, lactate dehydrogenase (both from rabbit muscle, salt-free), and V8 staphylococcal protease were from Sigma. Ornithine transcarbamylase was purified partially, free of CK, from _E. faecium_ according to Ref. 1. Hexokinase and glucose-6-phosphate dehydrogenase (both from yeast) were from Roche Molecular Biochemicals. Enzymes were freed from peroxidase and monomer and were used for the CK assays by centrifugal gel filtration through Sephadex G-50 (19). Buffer pH values were determined at 22 °C. Goat anti-rabbit IgG or anti-mouse IgG conjugated with peroxidase were from Promega, ammonium carbamate was from Aldrich, dimethyl suberimidate was from Pierce, and polyethylene glycols were from Fluka or Hampton. Other reagents were of the highest quality available.

**Polymerase Chain Reaction Cloning of the _P. furiosus_ Gene for CPS—** Genomic DNA from _P. furiosus_ (a generous gift of Dr. F. E. Jenney, Jr., Dept. of Biochemistry, University of Georgia, Athens, GA) was used as a template for polymerase chain reaction amplification of the CPS gene. The polymerase chain reaction products were digested with _Sph_ I and _Nco_ I site downstream of the stop codon. The polymerase chain reaction products were digested with _Nco_ I and _Bgl_ II and inserted into the corresponding sites of plasmid pET-15b (Novagen) behind the T7 promoter using T4 DNA ligase (USB) followed by transformation of _E. coli_ DH5α. The CPS gene in the resulting plasmid called pCP184 was sequenced using an ABI prism DNA Sequenator (Applied Biosystems).

**Expression and Purification of Recombinant _P. furiosus_ CPS—** _E. coli_ BL21 (DE3) cells transformed with the plasmid pCP184 were grown at 37 °C in a shaking incubator in 3 liters of LB broth containing 0.1 mg/ml ampicillin until an _A_ 600 of 0.5 was reached. After a 3-h induction with 1 mM isopropyl β-D-thiogalactoside, the cells (6 g) were harvested by centrifugation, resuspended in 40 ml of 50 mM Tris-HCl, pH 7.5, at 4 °C, and disrupted by sonication. The recombinant CPS was purified in a low amount. Therefore, the pCP184-transformed BL21 (DE3) cells were transformed with the plasmid pJS1240 (a plasmid that incorporates the spectinomycin resistance gene from pSJS1240) into _E. coli_ BL21 (DE3) cells transformed with the plasmid pET-15b (Novagen) behind the T7 promoter using T4 DNA ligase (USB) followed by transformation of _E. coli_ DH5α. The CPS gene in the resulting plasmid called pSJS1240 was sequenced using an ABI prism DNA Sequenator (Applied Biosystems).

**RESULTS**

Expression and Characterization of Recombinant _P. furiosus_ CPS—The expected band of approximately 34 kDa (exact mass deduced from the gene sequence (14), 34.3 kDa) was detected by SDS-PAGE in low quantity (<5% protein) in extracts of _E. coli_ cells carrying the pCP184 plasmid but not in extracts of cells carrying the progenitor pET-15b plasmid without the in-
The sequence-deduced mass of the fragment that begins in residue 203 of the reported amino acid sequence deduced from the gene (Fig. 1) is in excellent agreement with the electrophoretic estimate. All these data confirm the fidelity of the polymerase chain reaction cloning strategy used to generate the recombinant enzyme. The equilibrium of the reaction

\[ \text{CPS} + 	ext{ATP} \rightarrow \text{CP} + 	ext{ADP} \]

typically represents 10% of full activity (15, 16). In contrast, the bicarbonate-dependent ATPase activity of CPSs typically represents 10% of full activity (15, 16).

Equilibrium of the Reaction—The equilibrium of the reaction catalyzed by CPS is fully displaced toward CP synthesis (see Ref. 5), whereas with CK the value of the \( K_{eq} \) (1) predicts conversion of only a small fraction of the ATP to ADP at the concentrations of carbamate expected to be present in the assay mixtures used here. The results illustrated in Fig. 3 confirm for \( E. coli \) CPS and enterococcal CK these expectations. With the former the amount of CP (Fig. 3) produced increases linearly with the amount of enzyme even when a large fraction of the ATP is used up, whereas with enterococcal CK the production of CP rapidly flattens out with increasing amounts of the

The pyrococcal enzyme was recognized in enzyme-linked immunosorbent assay tests and Western blots by polyclonal rabbit antiserum raised against the CK from \( E. faecium \), although approximately 50-fold higher concentrations of the antiserum were necessary to get the same response as with \( E. faecium \) CK (Fig. 2). Three monoclonal antibodies against \( E. faecium \) CK only mAbCK1, recognizing an epitope localized within the C-terminal end 14 residues of CK (6), cross-reacted with the pyrococcal enzyme (data not shown), although approximately 100-fold higher antibody concentrations were needed for an equal reaction as with enterococcal CK. The immunological cross-reactivity of the two enzymes reflects their relatedness; the differences in reactivity being compatible with the 49% sequence identity.

Stoichiometry of the Reaction and ATPase Activity—Table I compares the production of ADP, \( P_i \), and CP by the pyrococcal enzyme with those observed with typical CK and CPS in an assay mixture containing ATP, bicarbonate, and ammonia. Because these assays also included large amounts of ornithine and ornithine transcarbamylase to convert the CP produced to citrulline and phosphate, CK and CPS should yield in these assays, respectively, 1 and 2 mol of both \( P_i \) and ADP/mole of citrulline produced. The results obtained with enterococcal CK and \( E. coli \) CPS agree, within experimental error, with these expectations. With the pyrococcal enzyme the results, at both 37 and 60 °C (the ornithine transcarbamylase used for coupling is stable at 60 °C) (see Ref. 1), are essentially the same as with CK: a ratio of approximately 1 is found between the amounts produced of \( P_i \), ADP, and citrulline.

Previously the CPSs purified from \( P. abyssi \) (12) and \( P. furiosus \) (14) were reported to exhibit an ATPase activity in the absence of ammonia and in the presence of bicarbonate, corresponding to half of the ATP consumption in the presence of ammonia. When we assayed this ATPase activity with the recombinant enzyme at 37 and 60 °C, we did not detect such activity (Table I) unless the concentration of enzyme used was vastly increased (data not shown), corresponding to similarly low activity as with enterococcal CK (0.3% of full activity (1, 6)). In contrast, the bicarbonate-dependent ATPase activity of CPSs typically represents 10% of full activity (15, 16).

The pyrococcal enzyme was recognized in enzyme-linked immunosorbent assay tests and Western blots by polyclonal rabbit antiserum raised against the CK from \( E. faecium \), although approximately 50-fold higher concentrations of the antiserum were necessary to get the same response as with \( E. faecium \) CK (Fig. 2). Of three monoclonal antibodies against \( E. faecium \) CK only mAbCK1, recognizing an epitope localized within the C-terminal end 14 residues of CK (6), cross-reacted with the pyrococcal enzyme (data not shown), although approximately 100-fold higher antibody concentrations were needed for an equal reaction as with enterococcal CK. The immunological cross-reactivity of the two enzymes reflects their relatedness; the differences in reactivity being compatible with the 49% sequence identity.
Solutions containing 0.1 M Tris-HCl, pH 8.0 (assays at 37 °C) or 9.0 (assays at 60 °C), 5 mM ATP, 5 mM MgCl₂, 40 mM NaHCO₃, 6 mM L-ornithine, 25 units/ml ornithine transcarbamylase (free from ammonium sulfate), 0.2 mM NH₄Cl (or, when omitted, 0.2 mM KCl), and one of the following: 53 μg/ml E. coli CPS, 0.083 μg/ml enterococcal CK, or 2.5 μg/ml (37 °C), or 0.45 μg/ml (60 °C) pyrococcal CK-like CPS were incubated at the indicated temperature. The reactions were initiated with the addition of the enzyme and were terminated 15 min later by the addition of an equivalent volume of 15% trichloroacetic acid. The precipitated protein was removed by centrifugation, and Pi, citrulline, and ADP were determined in the supernatant.

| Enzyme       | Temperature °C | NH₄Cl | ADP (μM) | Pi (μM) | Citrulline (μM) |
|--------------|----------------|-------|----------|---------|----------------|
| CPS          | 37             | 0.2   | 2.80 (1.90) | 3.10 (2.11) | 1.47 (1.00) |
| CK           | 37             | 0.2   | 0.76 (0.84) | 0.90 (0.99) | 0.91 (1.00) |
|              | 37             | 0.2   | 0.00     | 0.00     | 0.00          |
| CK-like CPS  | 37             | 0.2   | 0.12 (0.99) | 0.13 (1.00) | 0.13 (1.00) |
|              | 37             | 0.2   | 0.00<sup>a</sup> | 0.00<sup>a</sup> | 0.00<sup>a</sup> |
|              | 60             | 0.2   | 0.29 (0.97) | 0.29 (0.97) | 0.30 (1.00) |
|              | 60             | 0.2   | 0.00     | 0.00     | 0.00          |

<sup>a</sup> An identical assay with 10-fold more enzyme (25 μg/ml) did not yield any detectable product.

The enzyme, despite the existence of high concentrations of ATP and large excesses of bicarbonate and ammonia assuring a constant concentration of carbamate. The results with the pyrococcal enzyme fully replicate those obtained with enterococcal CK, as expected if the reactions catalyzed by the pyrococcal and enterococcal enzymes are identical. Furthermore, the extent of the reaction with E. coli CPS monitored by the production of ADP (Fig. 4) was the same whether or not the CP formed was removed by coupling with ornithine transcarbamylase, whereas with both enterococcal CK and the pyrococcal enzyme, the addition of ornithine transcarbamylase greatly increased the production of ADP, as expected if the equilibrium were displaced in the forward reaction by the removal of the product CP.

Use of Carbamate as Substrate of the Pyrococcal Enzyme—CK phosphorylates carbamate (2, 3), whereas bicarbonate and ammonia are the true substrates of CPS (5). In agreement with this, Fig. 5 shows that the production of CP (determined as citrulline via coupling with ornithine transcarbamylase) by E. coli CPS is the same with fresh and aged mixtures of potassium carbonate and ammonium chloride, whereas with enterococcal CK substantially more citrulline is formed with the aged than with the fresh mixtures; for in the latter, at the moment of addition to the assay carbonate and ammonia have not yet equilibrated with carbamate (3). The results with the pyrococcal enzyme are similar to those with enterococcal CK, indicating that carbamate is also the substrate for the enzyme from P. furiosus. However, to observe differences with aged and fresh mixtures, the mixtures had to be diluted more in the case of the pyrococcal enzyme than with enterococcal CK.

Further proof of the use of carbamate by the pyrococcal enzyme was obtained (Fig. 6) by comparing the production of CP when either ammonium carbamate or ammonium carbonate was abruptly added to mixtures at pH 9.5 and 10 °C containing ATP and the enzyme. These conditions were used by Jones and Lipmann (2) to demonstrate the use of carbamate by E. faecalis CK, because at this pH and temperature the stability of carbamate is increased. Our results confirm (2) for enterococcal CK and demonstrate for the pyrococcal enzyme, which is also active and stable at pH 9.5 (data not shown), the production of more CP with ammonium carbamate than with the carbonate. Again, as in the experiments with the fresh and aged mixtures reported in the previous paragraph, lower concentrations of carbamate and carbonate had to be used with the pyrococcal than with the enterococcal enzyme to demonstrate the differences. In summary, the results with fresh and aged solutions of ammonium carbonate and the results using carbonate or carbamate concur by showing that the pyrococcal enzyme uses carbamate rather than bicarbonate and ammonia as the substrate of the reaction.

Phosphorylation of ADP by Carbamoyl Phosphate—The established function in vivo of CK is to synthesize ATP from ADP and CP. In agreement with this function, enterococcal CK catalyzes faster the phosphorylation of ADP than the synthesis of CP (1). In contrast, CPSs do not catalyze the full reversion of their reaction of CP synthesis (5, 15, 35), although they catalyze, as a partial reverse reaction occurring at a rate of only 20% of the full reaction (5, 15), the synthesis of one molecule of ATP from one molecule of ADP and CP. Table II confirms that enterococcal CK catalyzes faster the phosphorylation of ADP than the synthesis of CP. Under the conditions of the assays illustrated in the Table the rate of ATP formation by this enzyme is approximately 4-fold higher than the rate of CP synthesis. Table II also shows that the pyrococcal enzyme catalyzes actively, as expected for a CK, the formation of ATP from ADP and CP, although in this case the ratio between the forward and reverse reactions approximates unity, possibly reflecting the adaptation of this enzyme to the new function of making CP rather than using it.

Structural Similarity with Enterococcal CK Revealed by X-ray Studies—Crystals of the pyrococcal enzyme grown in the absence or presence of MgATP (Fig. 7) diffracted with a conventional x-ray source to at least 2.6 and 2.0 Å resolution, respectively, although spectra were collected at 2.9 Å (98% completeness; R<sub>merge</sub>, 8.4) without ATP and at 2.2 Å (95.6% completeness; R<sub>merge</sub>, 11.7) with MgATP. The space group is, for the crystal without substrates, tetragonal P4 with unit cell parameters a = b = 97.78 Å and c = 135.42 Å. Packing density considerations (36) indicate that for a monomer mass of 34.3 kDa the unit cell could contain 16 monomers (V<sub>solvent</sub>, 2.35 Å<sup>3</sup>/Da; solvent content, 47%), corresponding to four monomers in the asymmetric unit. Cross-linking with dimethyl suberimidate (Fig. 8) confirms (14) that the enzyme is dimeric and reveals the formation of dimers of dimers. Upon treatment with dimethyl suberimidate a major band appears corresponding to

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*<sup>2</sup> Preliminary estimates yield an approximate K<sub>m</sub> for carbamate of 7 μM. This value is about 10-fold lower than the K<sub>m</sub> for carbamate of enterococcal CK (1). The concentrations of carbamate were calculated from the equilibrium with bicarbonate and ammonia given in Ref. 1.*
the dimer and a less prominent band with the mass of the tetramer is also seen, whereas the cross-linking of three monomers is detectable but less frequent. Thus, a dimer of dimers is likely to occupy the asymmetric unit of the crystal. Cross-linking of enterococcal CK under the same conditions (Fig. 8) confirms its dimeric character (1) and reveals lesser tendency to form dimers of dimers. In the presence of MgATP space group was orthorhombic P2_12_12 with unit cell parameters a = 55.22 Å, b = 90.92 Å, and c = 132.93 Å and an estimated number of 8 monomers/unit cell (V_m = 2.53 Å^3/Da; solvent content, 51%) or two monomers, possibly making a dimer, in the asymmetric unit.

Given the existence of nearly 50% sequence identity between the pyrococcal enzyme and enterococcal CK (6), and because we have recently determined the three-dimensional structure of CK enterococcal by x-ray crystallography (11), the molecular replacement method (37) was used to attempt phasing of the observed structure factors from the coordinates of enterococcal CK. For the spectra of the crystals grown in the absence or in the presence of MgATP the results are unambiguous with
values of the rotation and translation solutions exceeding consistently the values of all other peaks, which strongly indicates that the correct orientation of the model was determined in the

\[ \text{rotation and translation solutions} \]

**DISCUSSION**

The present results clearly show that the CP-synthesizing activity previously reported in *P. furiosus* (13, 14) is due to an enzyme that uses chemically made carbamate and a single ATP molecule to synthesize CP reversibly. The reaction exhibits the characteristic equilibrium of the CK reaction, an equilibrium that does not favor at 37 °C the accumulation of CP. The enzyme catalyzes with comparable efficiency the forward and reverse reactions, as shown in Table II.

| Enzyme           | Rate (μmol/min mg) | ATP/ADP ratio |
|------------------|--------------------|---------------|
| Pyrococcal CK-like CPS | 0.98 ± 0.04       | 1.05 ± 0.04   | 1.07          |
| Enterococcal CK   | 557 ± 3           | 2325 ± 71     | 4.17          |

* Result from a single assay.

**Fig. 6.** Comparison of carbamoyl phosphate synthesis from ammonium carbamate and ammonium carbonate. Each tube contained 0.1 M glycine, pH 9.5, 7 mM MgCl₂, 8.8 mM ATP, and 0.02 or 2 mg/ml enterococcal CK or CK-like CPS, respectively. The open circles represent the tube to which 136 μmol/ml solid ammonium carbamate (enterococcal CK) or 13.3 μmol/ml ammonium carbonate added as a 1.33 M freshly made solution at 0 °C in assay buffer (CK-like CPS) were added at zero time. The solid circles represent the tubes to which solutions of potassium carbonate (134 or 13.4 μmol/ml CK or CK-like CPS reaction medium, respectively) and ammonium chloride (268 or 26.8 μmol/ml CK or CK-like CPS reaction medium, respectively) were added. The incubation was at 10 °C. The total volume was 1 ml, of which samples were taken at the intervals noted for Pi measurement after alkaline decomposition of CP with 0.35 N NaOH.

**Fig. 7.** Crystals of the recombinant pyrococcal CK-like CPS of about 0.4 mm maximum dimension grown in the absence of ATP (left panel) or of about 0.7 mm grown in the presence of 20 mM MgATP (right panel) seen under polarized light.

**Fig. 8.** Cross-linking of pyrococcal CK-like CPS or of enterococcal CK with dimethyl suberimidate after the procedure of Davies and Stark (34). The indicated enzyme was incubated 3 h at 22 °C in 0.2 M triethanolamine HCl, pH 8, with (DMS) or without (−) 3 mg/ml freshly dissolved dimethyl suberimidate. Samples of the incubations were subjected to SDS-PAGE in 5% acrylamide/0.133% bisacylamide gels using a phosphate-based buffer system. The number of monomers in the various oligomeric forms are indicated at the right margin by the number preceding the monomer (M). st, protein standards (SDS-6H, Sigma).

two cases (see “Experimental Procedures”). The solution corresponds to dimers with the same overall shape of the enterococcal CK dimer packed in the crystal without interference between different dimers (Fig. 9). In contrast, a similar study made with a polyalanine model of biotin carboxylase, which is the basic structure of the catalytic domains of the typical, high molecular weight CPS (10), failed to yield any unambiguous solution, indicating that the folding of the CPS from *P. furiosus* resembles much more CK than biotin carboxylase and, by extension, typical CPSs. In addition, the organization of the monomers in the dimer given by the solution obtained with the CK monomer does not resemble the subunit organization in the quaternary structure of biotin carboxylase (38).
reverse reactions, and it is very inefficient in phosphorylating bicarbonate instead of carbamate, judging from its very small bicarbonate-dependent ATPase activity. All these properties are shared by typical CKs, such as the enterococcal enzyme (1, 2, 6). Our results are in conflict with the previously reported stoichiometry of 2 mol of ADP released/mole of CP formed by the partially purified enzyme from *P. furiosus*, assayed at 37 °C (14) or by the enzyme isolated from *P. abyssi*, assayed at 27 °C (12). Because these earlier enzyme preparations exhibited much greater ATPase activity in the absence of ammonia than the highly purified recombinant *P. furiosus* enzyme used here, the discrepancy would be explained if there were contaminating ATPases in the previous preparations that might have led to overestimation of the ADP production associated with CP synthesis. This possibility would be rigorously excluded with enzyme preparations obtained from cultures of pyrococci. Alternatively, it might be speculated that the highly purified enzyme used here is an individual component of a multicomponent CPS that would exhibit the classical CPS stoichiometry of 2 mol of ADP/mole of CP and that would be present in the possibly less pure preparations previously obtained from pyrococci (12, 14). However, this possibility is not supported by the similar specific activity and homodimeric nature of the recombinant and naturally produced enzymes and it also makes little biological sense, because the sole practical result of using such complex machinery would be to use an extra ATP molecule/mole of citrulline made in the ornithine transcarbamylase-coupled reaction. The coupling with ornithine transcarbamylase is essential in pyrococci given the rapid decomposition of CP at high temperature (13).

The high degree of sequence identity and immunological cross-reactivity of the pyrococcal enzyme and the enterococcal CK confirm the similarity of the two enzymes. Furthermore, our initial structural data obtained by x-ray crystallography clearly show that the pyrococcal enzyme exhibits a three-dimensional structure and quaternary organization that are highly similar to those of enterococcal CK and that are very different from those of CPS. Although the CK structure (11) reveals the existence of two catalytic sites/enzyme dimer, the relative orientation of the sites and the absence of intramolecular tunnels joining them exclude the possibility of catalytic collaboration between the two sites that is required for the synthesis of CP from bicarbonate and ammonia in three steps (bicarbonate phosphorylation, carbamate formation, and carbamate phosphorylation) that characterizes the mechanism of CPS (10, 39). In summary, all indicate that except for its extreme thermostability and low activity at normal temperatures, the pyrococcal enzyme is endowed with the characteristics of classical CKs. The finding of similar enzymes in *P.*

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3 However, the activity at 100 °C of the *P. furiosus* enzyme may be similar to that of enterococcal CK at 37 °C. Thus, the pyrococcal enzyme released at 95 °C, under the conditions of the CK assay (1), approximately 250 μmol P_i min⁻¹mg protein⁻¹, whereas the enterococcal CK produces at 37 °C in the same assay approximately 600 μmol citrulline·min⁻¹mg protein⁻¹ (1).
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abysii (12) and Pyrococcus horikoshii (a gene has been found in this organism (GenBankTM accession number 32577702) encoding a putative polypeptide exhibiting 89% sequence identity with the enzyme from *P. furiosus*) strongly suggests that this enzyme constitutes a constant component of the catalytic machinery of the hyperthermophiles of the *Pyrococcus* genus.

As already indicated, the generally accepted function of CK is to synthesize ATP from ADP and the CP produced mainly in the catabolism of arginine by the arginine deiminase pathway (4). There are strong reasons to deny such a function for the catabolism of arginine by the arginine deiminase pathway is to synthesize ATP from ADP and the CP produced mainly in the extracts of *P. furiosus* cells cultivated in a medium containing 0.2 g arginine liter\(^{-1}\) (40), suggesting that the arginine deiminase pathway is not operative in this organism. In keeping with this, no arginine deiminase putative gene was identified in the entire genome of the related organism *P. horikoshii* (41). If such a gene had existed in *P. horikoshii*, it should have been identified given the constant sequence motifs that are characteristic of arginine deiminases (42). *P. furiosus* cells could be grown in a defined medium containing ornithine as an arginine precursor (40), and the enzymes of the biosynthetic pathway of arginine, anabolic ornithine transcarbamylase, argininosuccinase synthetase, and argininosuccinase were detected in this organism (43). Similarly, aspartate transcarbamylase, the enzyme that catalyzes the second step of pyrimidine biosynthesis, was detected in both *P. furiosus* (13) and *P. abyssi* and was characterized in the latter organism (44). Thus, CP has to be made in these microorganisms to be utilized by ornithine transcarbamylase and aspartate transcarbamylase in the biosynthesis of arginine and pyrimidines. The only CP-making activity detected in extracts from these microorganisms was due to the enzyme studied here (12, 13). This enzyme appears to be coupled functionally and to form physical complexes with ornithine transcarbamylase (13) and aspartate transcarbamylase (45) for there is evidence of efficient channeling of the CP in the direction of citrulline and carbamoyl aspartate biosynthesis.

The inexistence in these organisms of classical CPS is also supported by the lack of detection of a classical CP gene in the full genome of *P. horikoshii* (41). Again, it would be unlikely that such a gene would have escaped detection because many constant regions of characteristic sequence exist in all classical CPs (9), and, for example, classical CP genes were detected (whereas no CK genes were detected) in the genomes of the other three archaea that have been sequenced fully, *Methanothermobacter jannaschii* (46), *Archaeoglobus fulgidus* (47), and *Methanobacterium thermoautotrophicum* (48). Taken together, all these data strongly suggest that the CK studied here plays a new metabolic role: the biosynthesis of CP for anabolic purposes.

Such an extraordinary use of a CK appears to be rendered possible by the extreme living conditions of pyrococci. Whereas in the mesophilic world the chemical formation of carbamate (49) might be slower than required by the needs of CP, rendering essential the enzymatic formation of carbamate by CPS in the initial two steps of its reactional mechanism (39), the high temperature in hydrothermal vents assures rapid chemical formation of carbamate without the need for enzyme catalysis. Another function fulfilled by mesophilic CPS is the provision of a high local concentration of carbamate at the site of carbamate phosphorylation within the enzyme. This becomes possible by coupling the synthesis of carbamate with the cleavage of an extra ATP molecule. However, the enzymatic synthesis of high local concentrations of carbamate appears unnecessary in *P. furiosus* given the high affinity of the pyrococcal enzyme for carbamate,2 particularly because the concentration of carbamate may be relatively high in the habitat of *P. furiosus* given the finding in hydrothermal vents of high concentrations of CO\(_2\) (50) (the true reactant, rather than bicarbonate, in the chemical synthesis of carbamate (49)) and of 0.6–1 mM ammonia possibly derived from sediments (51) similar to those from where *P. furiosus* was grown (52). In fact, the enzyme activity in *P. furiosus* appears more than enough to serve the needs of arginine and pyrimidine synthesis, even at suboptimal concentrations of carbamate. Thus, from the increase in the enzyme activity given in Ref. 13, when the assay temperature is raised from 60 to 90 °C, the activity in the initial *P. furiosus* extract would be at 90 °C approximately 10 \(\mu\)mol h\(^{-1}\) mg protein\(^{-1}\) (14), a value that is 9-fold higher than the activity of CPS in *E. coli* extracts, assayed at 37 °C (16). Another characteristic of the CPS reaction that is mimicked by the high temperature of the habitat of pyrococci is the irreversibility of the synthesis of CP, because the rapid decomposition of CP at 100 °C (13) would cause the concentration of this product to be essentially nil, thus displacing strongly the reaction in the direction of CP synthesis. In summary, the extreme living conditions of the pyrococci may render unnecessary the enzymatic synthesis of carbamate by CPS with the associated expenditure of an extra ATP molecule, as a prelude to making CP, explaining the use of CK for CP synthesis in these organisms. It is of interest that CK appears to have become adapted in *P. furiosus* to its new anabolic function, because when compared with enterococcal CK, it exhibits greater apparent affinity for carbamate and is less effective in the synthesis of ATP from ADP and CP, relative to CP synthesis. Other adaptations exhibited by the pyrococcal CK that deserve further study are the much lower specific activity at 37 °C and much higher thermal stability than classical CK. Detailed comparisons of the three-dimensional structures of the enterococcal and pyrococcal CKs and site-directed mutagenesis of key residues in the two enzymes will be essential to ascertain the reasons for these differences. Experiments with these goals are currently in progress in our laboratory.

Acknowledgments—We thank Drs. Francis E. Jenney, Jr. (Dept. of Biochemistry, University of Georgia, Athens, GA) for giving us genomic DNA from *P. furiosus*, S. J. Sandler (Dept. of Microbiology, University of Massachusetts) for providing pJS1240, J. Cervera (Fundación Valenciana de Investigaciones Biomédicas, Valencia) for the gift of *E. coli* CPS and the monoclonal antibodies against CK, E. Grau (Instituto de Biología Molecular y Celular de Plantas, CSIC, Valencia) for automated DNA sequencing, and the Servicio de Química de Proteínas del Centro de Investigaciones Biológicas (CIB-CSIC, Madrid) for N-terminal protein sequencing.

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