Characterization of the Aspartate Transcarbamoylase from Methanococcus jannaschii

Received for publication, November 17, 1999, and in revised form, March 17, 2000
Published, JBC Papers in Press, March 19, 2000, DOI 10.1074/jbc.M909220199

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The genes from the thermophilic archaeabacterium Methanococcus jannaschii that code for the putative catalytic and regulatory chains of aspartate transcarbamoylase were expressed at high levels in Escherichia coli. Only the M. jannaschii PyrB (Mj-PyrB) gene product exhibited catalytic activity. A purification protocol was devised for the Mj-PyrB and M. jannaschii PyrI (Mj-PyrI) gene products. Molecular weight measurements of the Mj-PyrB and Mj-PyrI gene products revealed that the Mj-PyrB gene product is a trimer and the Mj-PyrI gene product is a dimer. Preliminary characterization of the aspartate transcarbamoylase from M. jannaschii cell-free extract revealed that the enzyme has a similar molecular weight to that of the E. coli holoenzyme. Kinetic analysis of the M. jannaschii aspartate transcarbamoylase from the cell-free extract indicates that the enzyme exhibited limited homotropic cooperativity and little if any regulatory properties. The purified Mj-catalytic trimer exhibited hyperbolic kinetics, with an activation energy similar to that observed for the E. coli catalytic trimer. Homology models of the Mj-PyrB and Mj-PyrI gene products were constructed based on the three-dimensional structures of the homologous E. coli proteins. The residues known to be critical for catalysis, regulation, and formation of the quaternary structure from the well characterized E. coli aspartate transcarbamoylase were compared.

Organisms from the archaea, prokaryota, and eukarya kingdoms all produce aspartate transcarbamoylase, the enzyme that catalyzes the committed step of the pyrimidine biosynthetic pathway, the reaction of carbamoyl phosphate and L-aspartate to form N-carbamoyl-L-aspartate and inorganic phosphate (1). There are four major classes or forms of quaternary structures known for aspartate transcarbamoylases. In prokaryotes, aspartate transcarbamoylase is known to exist in three classes. The simplest is class C, a homotrimer of catalytic chains each with a molecular mass of approximately 34 kDa. The aspartate transcarbamoylase from Bacillus subtilis, which lacks both homotropic and heterotropic properties, is an example of this class (2). A second form of aspartate transcarbamoylase, class A, is a dodecamer of six 34-kDa and six 45-kDa polypeptides. Catalytic and regulatory functions of this enzyme are both located on the 34-kDa polypeptides, whereas the function of the 45-kDa polypeptides is unknown. There are several species of Pseudomonas that produce this type of aspartate transcarbamoylase, including Pseudomonas fluorescens (3, 4). The third and best characterized class of aspartate transcarbamoylase is class B, comprised of two trimeric catalytic subunits of 34-kDa polypeptides and three dimeric regulatory subunits of 17-kDa polypeptides. The class B form is an allosteric enzyme, exhibiting both homotropic and heterotropic interactions. Escherichia coli, Salmonella typhimurium, Erwinia herbicola, Serratia marcescens, and other members of the family Enterobacteriaceae produce class B aspartate transcarbamoylase (5). In eukaryotes, aspartate transcarbamoylase often is a part of a multienzyme complex, such as those found in yeast (6) and hamster (7).

The DNA sequence of the unicellular thermophilic archaeabacterium Methanococcus jannaschii revealed genes (pyrB and pyrI) that are homologous to a class B aspartate transcarbamoylase (8). In the E. coli genome, the pyrB and pyrI genes are separated by only 12 base pairs, whereas in the M. jannaschii genome the two homologous genes are separated by over 200,000 base pairs. Fig. 1 shows a sequence alignment of the E. coli and M. jannaschii pyrB and pyrI genes. The PyrB gene product exhibits 47% identity and 67% similarity, and the PyrI gene product exhibits 35% identity and 52% similarity, suggesting that the PyrB and PyrI gene products of the two species have similar tertiary structures. The M. jannaschii differs from other recently characterized archaeabacteria such as Pyrococcus abyssi (9) and Sulfolobus acidocaldarius (10) that contain an enterobacteria-like pyrBI operon.

In order to study the M. jannaschii aspartate transcarbamoylase, the M. jannaschii pyrB (Mj-pyrB) and M. jannaschii pyrI (Mj-pyrI) genes were inserted into an expression system that yielded substantial amounts of both gene products. A protein purification scheme was developed for the Mj-PyrB and Mj-PyrI gene products, and the quaternary structure of the M. jannaschii aspartate transcarbamoylase found in vivo was analyzed and kinetically characterized.

EXPERIMENTAL PROCEDURES

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Materials—Agarose, ATP, CTP, L-aspartate, N-carbamoyl-L-aspartate, 2-mercaptoethanol, isopropyl-β-thiogalactosidase, potassium di-
hydrogen phosphate, sucrose, phenylmethylsulfonyl fluoride, bovine pancreatic deoxyribonuclease I, and uracil were obtained from Sigma. Q-Sepharose Fast Flow and high performance phenyl-Sepharose resin were purchased from Amersham Pharmacia Biotech. Ampicillin and the Sequenase DNA sequencing kit were obtained from United States Biochemical Corp. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs.

Sodium dodecyl sulfate, Bio-Prep SE-1000/17, Bio-Prep SE-100/17, gel filtration molecular weight standards, and the Protein Assay Dye were purchased from Bio-Rad. Pefabloc SC, pepstatin, and leupeptin were purchased from Roche Molecular Biochemicals. Carbamoyl phosphate dilithium salt, obtained from Sigma, was purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at −20 °C (11). Casamino acids, yeast extract, and tryptone were obtained from Difco. Glass beads for isolation of DNA from agarose gels were purchased from Bio 101. Ammonium sulfate, urea, Tris, and electrophoresis-grade acrylamide were purchased from ICN Biomedicals. An-tipyrine was obtained from Fisher. Oligonucleotides were purchased from Operon Technologies. The pGEM T-tail vector kit was purchased from Promega.

**Strains and Plasmids**—The *E. coli* strains 627239 and 623859 harboring plasmids AMJK64 and AMJAE67, respectively, were obtained from the American Type Culture Collection (ATCC). The plasmid pET23a was obtained from Novagen, Inc., and the plasmid pSJS1240 was provided by S. Sandler.

**Construction of Plasmids for the Expression of the Mj-PyrB and Mj-PyrI Gene Products**—DNA encoding the Mj-pyrB and Mj-pyrI genes were derived from plasmids AMJK64 and AMJAE67, respectively (8). Each plasmid was incubated with EcoRI and BamHI restriction endonucleases, and the corresponding EcoRI-BamHI DNA fragments containing the *Mj-pyrB* and *Mj-pyrI* genes were separated by agarose gel electrophoresis followed by isolation from the gel with glass beads. Two sets of oligonucleotide primers were used to amplify the *Mj-pyrB* and *Mj-pyrI* gene fragments by PCR (12). One set of primers introduced a unique NdeI restriction site overlapping the 5' initiation codon and the other a unique SacI restriction site proximal to the 3' end of the termination codon. The PCR products were separated by agarose gel electrophoresis followed by isolation of the appropriate fragment from the gel with glass beads. Each fragment was separately mixed with linear p-GEM-T and treated with T4 DNA ligase for 16 h at 4 °C. After selection, plasmids pGEM-pyrB and pGEM-pyrI harboring the *Mj-pyrB* and *Mj-pyrI* genes, respectively, were obtained.

In order to express the recombinant genes under the control of the *E. coli* aspartate transcarbamoylase promoter (13), the *Mj-pyrB* and *Mj-pyrI* genes were transferred into plasmid pEK164 (14). In order to construct these plasmids, pGEM-pyrB, pGEM-pyrI, and pEK164 were digested with the restriction endonucleases NdeI and SacI. The DNA fragments containing the *Mj-pyrB* gene, *Mj-pyrI* gene, and pEK164 vector were isolated after agarose gel electrophoresis using glass beads. The isolated *Mj-pyrB* and *Mj-pyrI* gene fragments were individually mixed with the isolated vector fragment and treated with DNA ligase for 16 h at 4 °C. The resulting plasmids, pEK400 and pEK401, were confirmed by restriction analysis. DNA sequence analysis was then used to confirm that the PCR-amplified *M. jannaschii pyrB* and *pyrI* genes had exactly the same sequence as reported (8).

The *Mj-pyrB* and *Mj-pyrI* genes were also inserted into the expression vector pET23a, as an alternate expression system, employing the T7 promoter (15), could be tested. Plasmids pEK400, pEK401, and pET23a were digested with NdeI and SacI endonucleases to remove the *Mj-pyrB* and *Mj-pyrI* genes. These DNA fragments were separated by agarose gel electrophoresis, isolated using glass beads, and then were individually mixed with NdeI- and SacI-digested pET23a. The mixtures were then treated with T4 DNA ligase for 16 h at 4 °C. The resulting plasmids, pEK406 and pEK407, contained the *M. jannaschii* pyrB and pyrI genes respectively, under control of the T7 promoter.

**Expression of the Mj-PyrB and Mj-PyrI Gene Products**—Protein expression was performed with the plasmid transformation strain EK1104 (16) that has an inducible expression of these proteins (18). Typically, a 50-ml overnight culture of M9 media (19) supplemented with 0.5% casamino acids, 100 μg/ml ampicillin, and 100 μg/ml spectinomycin (when the plasmid pSJS1240 was used) was incubated with the appropriate plasmid/strain combination and grown overnight at 37 °C. A 2% inoculum was used to seed each of the four 4-liter flasks containing 2 liters of M9 media supplemented as indicated above (8 liters total). The cells were grown at 37 °C, 200 rpm to an A600 of 0.6 (normally 2.5 h after inoculation). Protein expression was then induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.4 mM. The cell culture was grown for 4 additional h before harvesting.

**Purification of the M. jannaschii PyrB Gene Product**—The cell culture was centrifuged at 4500 × g for 20 min, and the cell pellet was resuspended in 64 ml of ice-cold 0.1 M Tris-Cl buffer, pH 9.2. After sonication to lyse the cells, the mixture was centrifuged at 31,000 × g for 20 min. In order to increase protein yield, the cell pellet was resuspended in 32 ml of ice-cold 0.1 M Tris-Cl buffer, pH 9.2, and again centrifuged as described above.

The cell-free supernatant and the supernatant from the pellet wash were combined and brought to 30% saturation of ammonium sulfate. Aliquots of 5 ml were transferred into 18 × 150-mm test tubes and placed in a metal rack. This rack was then immersed in a 90 °C water bath and heated for 15 min after the mixture reached 90 °C (about 3 min). During the incubation at 90 °C, the tubes were gently shaken at 5-min intervals. This procedure was used to ensure that each aliquot

![Fig. 1. Sequence alignment of the E. coli (Ecoli) and M. jannaschii ( METJA) PyrB and PyrI gene products.](image-url)
reached 90 °C rapidly. After heating, the aliquots were recombined and centrifuged at 31,000 × g for 15 min. The supernatant was retained and dialyzed 2 times in 4 liters of 0.05 mM Tris acetate buffer, 2 mM 2-mercaptoethanol, pH 8.3.

The dialyzed supernatant was loaded onto a Q-Sepharose Fast Flow anion exchange column (1.8 × 18 cm) pre-equilibrated with 300 ml of column buffer, 0.05 mM Tris acetate, 2 mM 2-mercaptoethanol, pH 8.3. The protein was eluted from the column using 300 ml of column buffer followed by a 600-ml linear gradient of column buffer to column buffer containing 0.5 mM NaCl. The column fractions containing the Mj-PyrB gene product, as determined by A_{280} measurements and by SDS-PAGE, were pooled and dialyzed into a buffer solution containing 0.05 M Tris acetate, 2 mM 2-mercaptoethanol, and 1.3 mM ammonium sulfate, pH 7.0. The final purification step involved the use of hydrophobic interaction chromatography employing a phenyl-Sepharose column (1 × 28 cm) that was pre-equilibrated with a buffer solution of 40 mM KH₂PO₄, 2 mM 2-mercaptoethanol, and 1.3 mM ammonium sulfate, pH 7.0. The protein was loaded onto the column and washed with the same buffer solution to remove any unbound proteins. The protein was then eluted using a 110-ml linear gradient of 40 mM KH₂PO₄, 2 mM 2-mercaptoethanol, 1.3 mM ammonium sulfate, pH 7.0, to 40 mM KH₂PO₄, 2 mM 2-mercaptoethanol, pH 7.0. Fractions containing greater than 98% pure protein, as determined by SDS-PAGE, were pooled and concentrated.

**Purification of the M. jannaschii PyrI Gene Product**—A 2-liter cell culture was grown at 45 °C, 4% N₂/96% H₂, at 5,500 rpm, and the pellet was resuspended in 16 ml of ice-cold 0.1 M Tris-Cl, 0.1 mM zinc acetate buffer, pH 9.2. After sonication to lyse the cells, the mixture was centrifuged at 31,000 × g for 20 min. The supernatant was brought to 65% saturation of ammonium sulfate, after which it was centrifuged at 31,000 × g for 15 min. The pellet was dissolved and dialyzed 2 times in 4 liters of 40 mM KH₂PO₄, 2-mercaptoethanol, 0.1 mM zinc acetate buffer, pH 7.0. The dialyzed supernatant was again brought to 65% saturation of ammonium sulfate and centrifuged at 31,000 × g for 15 min. The pellet was dissolved and dialyzed 2 times in 4 liters of 0.05 mM Tris acetate, 0.1 mM zinc acetate, 2-mercaptoethanol, pH 8.3. The dialyzed supernatant was then loaded onto a Source-Q anion exchange column (1 × 6.5 cm) that was pre-equilibrated with a buffer solution of 0.05 mM Tris acetate, 0.1 mM zinc acetate, 2-mercaptoethanol, pH 8.3. The column was then eluted with the same buffer solution to remove any unbound proteins. The Mj-PyrI gene product was then eluted using a 32.5-ml linear gradient of 0.05 mM Tris acetate, 0.1 mM zinc acetate, 2-mercaptoethanol, pH 8.3, to 0.5 mM NaCl, 0.05 mM Tris acetate, 0.1 mM zinc acetate, 2-mercaptoethanol, pH 8.3. The fractions containing greater than 98% pure protein, as determined by SDS-PAGE, were pooled.

**Homology Modeling of Mj-PyrB and Mj-PyrI Gene Products**—The molecular weights of the Mj-PyrB and Mj-PyrI gene products were determined by gel filtration using a set of molecular weight standards. A Bio-Prep SE-1000/17 column buffer (30 cm) was used for the Mj-PyrI gene product. The SE-1000/17 column was equilibrated with 50 mM Tris acetate buffer, 2 mM 2-mercaptoethanol, pH 7.0. A standard was then resubmitted to Swiss-Model using the “First Approach” mode. The homology modeling template was the Protein Data Bank code 1D09, which is the recent 2.1-Å structure of aspartate transcarbamoylase in the presence of the bisubstrate analog N-phosphonacetyl-L-aspartate (31). The A and B chains of code 1D09 were used to model the Mj-PyrB and Mj-PyrI gene products, respectively. The initial model created using the “First Approach” mode was examined using the Swiss-Prot model viewer, and adjustments were made to the sequence alignment to improve the alignment of residues known to be functionally important in the corresponding E. coli protein. The model was then resubmitted to Swiss-Model using the “Optimize” mode. Final analysis of the models was performed in QUANTA (Molecular Simulations, Inc.).

**RESULTS AND DISCUSSION**

**Construction of Plasmids for the Expression of the Mj-pyrB and Mj-pyrI Genes**—Two separate approaches were taken in order to express the Mj-PyrB and Mj-PyrI gene products. The first was to place the M. jannaschii genes under the control of the E. coli pyrBI promoter in an approach similar to that used to express the B. subtilis aspartate transcarbamoylase in E. coli (13). The second was to place the M. jannaschii genes under the control of the bacteriophage T7 promoter using the pET plasmid system of Studier et al. (15).

**DNA Encoding the Mj-pyrB and Mj-pyrI Genes**—The DNA encoding the Mj-pyrB and Mj-pyrI genes were isolated from plasmids AMJPK84 and AMJAE87, respectively (8). Since both of the expression systems require a NdeI site at the initiation DMet codon, the required NdeI site as well as an additional restriction site (SacI) after the 3′ end of the coding region were introduced using PCR (12). The NdeI and SacI sites allowed easy cloning of the Mj-pyrB and Mj-pyrI genes into both the pyrBI and pET expression systems (see “Experimental Procedures” for details).
rare tRNA pools thus provides a useful method for overproduc-
the pET expression system along with the enhancement of the rare codons found in each of the two genes. The combination of PyrI gene product was enhanced little if at all (see Fig. 2). This expression of the Mj-PyrB gene product, whereas the Mj-
are 31 of these rare codons, whereas in the case of the M. jannaschii gene there was only 9. When the plasmid pSJS1240, encoding the argU and ileX tRNAs, was introduced into the pET Mj-pyrB and Mj-pyrI expression strains, there was a considerable improvement in the expression of the Mj-PyrB gene product, whereas the Mj-PyrI gene product was enhanced little if at all (see Fig. 2). This difference may be directly related to the relative numbers of rare codons found in each of the two genes. The combination of the pET expression system along with the enhancement of the rare tRNA pools thus provides a useful method for overproduc-
tion of both the Mj-PyrB and Mj-PyrI gene products in E. coli. Catalytic Activity of the Mj-PyrB and Mj-PyrI Gene Products—In order to determine whether the Mj-PyrB or Mj-PyrI gene products exhibited aspartate transcarbamoylase activity, crude cell extracts were analyzed using the highly sensitive colorimetric assay (25). Crude cell extracts containing the Mj-PyrB gene product expressed in E. coli exhibited considerable aspartate transcarbamoylase activity (data not shown). In extracts of cells without the plasmid containing the Mj-pyrB gene or with the Mj-pyrI gene, no aspartate transcarbamoylase activity could be detected (data not shown). These data indicate that the Mj-PyrB gene product is sufficient for aspartate transcarbamoylase activity, just as the corresponding PyrB gene product from E. coli is catalytically active even in the absence of the PyrI gene product.

Purification of the Mj-PyrB Gene Product—As seen in Fig. 3, lane B, the Mj-PyrB gene product is highly overexpressed in the presence of enhanced tRNA pools. A purification procedure for the Mj-PyrB gene product was developed taking advantage of the expected thermal stability of the protein. After the cells were broken open by sonication and centrifuged to remove cell debris, ammonium sulfate was added to 30% saturation followed by a 15-min, 90 °C heat treatment. Negligible loss of enzymatic activity and a 2-fold purification were observed (Fig. 3, lane C). The heat step was followed by two chromatography steps (Fig. 3, lanes D and E), the first employing anion exchange chromatography (Q-Sepharose Fast Flow) and the second employing hydrophobic interaction chromatography (phenyl-Sepharose). The overall protein purification procedure resulted in a 5.9-fold purification, negligible loss of enzyme activity, and greater than 98% pure Mj-PyrB gene product (see Table I and Fig. 3, lane E).

Thermal Stability of the M. jannaschii PyrB Gene Product—Given the fact that the optimal growth temperature of M. jannaschii is 85 °C as compared with 37 °C for E. coli, a comparison of the thermal stabilities of the PyrB gene products was carried out. As seen in Fig. 4, the M. jannaschii PyrB gene product is substantially more stable than the E. coli catalytic subunit. At 75 °C half of the initial catalytic activity of the E. coli catalytic subunit was lost in less than 1 min. On the other hand, the M. jannaschii PyrB gene product retained 75% of its activity after 60 min at the same temperature. The heat stability of the M. jannaschii PyrB gene product is very sensitive to the conditions used. In the heat step of the purification (90 °C) almost no reduction in catalytic activity was observed; however, the thermal stability of the enzyme was greatly enhanced by the addition of ammonium sulfate.

Quaternary Structure of the Mj-PyrB and Mj-PyrI Gene Prod-
TABLE I

| Step       | Protein concentration mg/ml | Activity a units mg | Total Activity units/mg | Purification fold |
|------------|-----------------------------|---------------------|-------------------------|-------------------|
| Sonicate   | 4.55                        | 4704                | 404                     | 11.5              |
| Heat       | 4.84                        | 4607                | 220                     | 20.9              |
| Q-Sepharose| 1.07                        | 3877                | 91                      | 42.7              |
| Phenyl-Sepharose | 1.24           | 4007                | 58                      | 69.1              |

a A unit of activity is defined as 1 μmol of carbamoyl aspartate formed per min. Activity measurements were carried out by the pH-stat method (25) at 25 °C with 30 mM aspartate and 4.8 mM carbamoyl phosphate.

FIG. 4. Comparison of the thermal stability of the M. jannaschii and E. coli PyrB gene products. Solutions of the M. jannaschii (○) and E. coli (○) PyrB gene products at 0.5 mg/ml were heated in 40 mM KH₂PO₄, 2 mM 2-mercaptoethanol, 0.2 mM EDTA, pH 7.0. Samples were removed at the indicated times and immediately chilled on ice. Enzymatic activity was determined by the colorimetric assay.

The genomic organizations of the Mj-pyrB and Mj-pyrI and the E. coli pyrB and pyrI genes differ substantially. In E. coli, the two genes are contiguous, separated by only 12 base pairs. In contrast, the Mj-pyrB and Mj-pyrI genes are separated by 200,000 base pairs of intervening sequence. Furthermore, the genomic organization of the Mj-pyrB and Mj-pyrI genes suggest that they are regulated by separate promoters, whereas the E. coli pyrB and pyrI genes are regulated as a single pyrBI operon. The striking differences in genomic organization between these two organisms are difficult to assess in terms of physiological or evolutionary significance. Indeed, it has not been determined whether the Mj-PyrB and Mj-PyrI gene products exist as monomers or higher order species, a molecular weight analysis was performed by gel filtration. The molecular masses of the Mj-PyrB and Mj-PyrI gene products were determined against a set of standards and found to be 129 and 32 kDa, respectively. The monomer molecular masses of the Mj-PyrB and Mj-PyrI gene products were determined by SDS-PAGE analysis to be 37 and 17 kDa, respectively. These values compared favorably with the theoretical molecular masses of 33.7 and 17 kDa as calculated from the primary amino acid sequences. These data indicate that the Mj-PyrB gene product associates as a trimer and that the Mj-PyrI gene product associates as a dimer. The trimeric structure of the Mj-PyrB gene product and the dimeric structure of the Mj-PyrI gene product is consistent with structures of other class B aspartate transcarbamoylases.

Quaternary Structure of M. jannaschii Aspartate Transcarbamoylase—The above data suggest that the M. jannaschii aspartate transcarbamoylase is organized in the class B form. To test this hypothesis, the quaternary structure of the M. jannaschii aspartate transcarbamoylase that exists in vivo was determined directly from an M. jannaschii cell-free extract, using sucrose density gradient sedimentation.

After centrifugation through the sucrose density gradient, the M. jannaschii cell-free extract was fractionated, and each fraction was assayed for aspartate transcarbamoylase activity. As standards, a mixture of the catalytic subunit and the holoenzyme of E. coli aspartate transcarbamoylase were centrifuged through an identical sucrose gradient, fractionated, and assayed for activity. As seen in Fig. 5, the peak aspartate transcarbamoylase activity in the M. jannaschii crude cell-free extract sedimented at a position almost identical to that of the E. coli holoenzyme. These results are consistent with the notion that the Mj-PyrB and Mj-PyrI gene products associate to form a quaternary structure similar to that observed for the class B aspartate transcarbamoylases, such as the E. coli holoenzyme.

In further support of this notion, preliminary experiments have demonstrated that mixing the purified Mj-PyrB gene product and partially purified Mj-PyrI gene product results in the formation of a quaternary structure similar in mass to the E. coli holoenzyme. However, the in vitro reconstituted M. jannaschii holoenzyme was extremely difficult to manipulate due to very limited solubility of the newly formed holoenzyme. Future experiments are planned to optimize conditions for the in vitro reconstitution of the M. jannaschii holoenzyme. Nevertheless, in vivo and in vitro studies indicate that the Mj-PyrB and Mj-PyrI gene products assemble to form an aspartate transcarbamoylase of the class B type.

Homotropic and Heterotropic Interactions of the M. jannaschii Aspartate Transcarbamoylase—Preliminary experiments were performed on the M. jannaschii aspartate transcarbamoylase partially purified by sucrose density gradient centrifugation from M. jannaschii cell extracts. Aspartate saturation curves at 37 °C revealed that at saturating concentrations of carbamoyl phosphate homotropic cooperativity with respect to aspartate was observed, although the value of the Hill coefficient was less than that observed for the E. coli enzyme. On the other hand, at subsaturating concentrations of
aspartate, ATP and CTP did not influence the activity of the enzyme (data not shown). Because of the crude enzyme preparation used, we consider these results tentative until a more highly purified preparation of the M. jannaschii aspartate transcarbamoylase can be tested. The extent of the homotropic and heterotropic interactions must also be evaluated at higher temperatures closer to the optimal growth temperature of M. jannaschii.

A Model of the Three-dimensional Structure of the Mj-PyrB and Mj-PyrI Gene Products—As seen in Fig. 1, the PyrB gene products from M. jannaschii and E. coli are extremely homologous, exhibiting 47% identity and 67% similarity, suggesting that they arose from a common ancestral gene. Residues determined to be critical for catalytic activity in the E. coli enzyme (32), including Ser92, Arg94, Thr95, Arg105, His134, Glu137, Arg167, Arg182, Glu211, Ser260, and Lys284 are all conserved in the Mj-PyrB gene product. As Ser280 and Lys284 are distributed into the active site of one catalytic chain from an adjacent catalytic chain in E. coli, the conservation of these residues suggests that the active site in the Mj-PyrB gene product is also at the interface between chains and is shared.

Interactions important for trimer stability such as the C1-C2 interchain interactions, Glu37–Lys40 (33), Arg54–Glu86 (34), Arg54–Tyr58, Arg65–Tyr68, Arg65–Asp100 (33), and Asp90–Arg269 (33) are all conserved. All residues involved in stabilization of the interface between two catalytic chains donated by different trimers, the C1-C4 interface, are conserved, including Lys164, Tyr165 (35), Glu233 (36), Arg234 (37), Glu239 (38), Tyr240 (39), and Asp271 (37).

The conservation of the Mj-PyrI gene product is slightly lower than that observed for the Mj-PyrB gene product with 35% identity and 52% similarity to the E. coli regulatory chain sequence (see Fig. 1). The residues that comprise the zinc-binding site, Cys139, Cys144, Cys138, and Cys141 are all conserved, strongly suggesting that this site is retained in the Mj-PyrI gene product. In the nucleotide effector binding site, all residues that interact with nucleotides via side chain interactions are conserved, including Asp19 (40), His20 (40), Lys80 (41), and Lys84 (42), suggesting that the Mj-PyrI gene product has a nucleotide-binding site.

In the interface between one catalytic chain in the top and one regulatory chain in the bottom of the molecule, the C1-R4 interface, the critical interface-stabilizing interaction between Lys143 (43) and Asp286 (44) is also conserved. In addition, many of the stabilizing interactions of the C1-R1 interface are also conserved. The only departure from interface residue conservation is in the R1-R6 interface, between the two regulatory chains of a dimer. Little of this interface is conserved; however, many of these interactions are backbone in nature and therefore do not rely on the nature of the specific side chains.

Fig. 6A is a three-dimensional model of the Mj-PyrB gene product derived from the x-ray structural data for one catalytic chain of the E. coli enzyme. Fig. 6B shows a three-dimensional model of the Mj-PyrI gene product derived from the x-ray structural data for one regulatory chain of the E. coli enzyme. The models of both the Mj-PyrB and Mj-PyrI gene products are overall extremely similar to the corresponding proteins from E. coli. Small insertions and deletions are located on surface loops such as the 20-, 80-, and 240-s loop of the Mj-PyrB gene product and the 30- and 130-s loops of the Mj-PyrI gene products. Since the 80- and 240-s loops of the E. coli catalytic chain are important for catalysis and homotropic cooperativity, the observed alterations in these loops may result in alterations in these properties of the M. jannaschii enzyme. The modeling of the PyrB and PyrI gene products as well as the conservation of functionally critical amino acid side chains suggest that the M. jannaschii and E. coli enzymes catalyze the transcarbamoylase.

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3 The residue numbering is based on the E. coli amino acid sequence.

4 Within the E. coli holoenzyme, the catalytic chains of the top catalytic trimer are numbered C1, C2, and C3, whereas the catalytic chains of the bottom catalytic trimer are numbered C4, C5, and C6, with C4 under C1. The regulatory dimers contain chains R1–R6, R2–R5, and R3–R6. A regulatory chain is in direct contact with the same numbered catalytic chain.
reaction by a similar mechanism and have a similar tertiary structure.

Comparison of the Amino Acid Sequence of the M. jannaschii and E. coli PyrB and PyrI Gene Products—There are several major differences in the amino acid composition of the *M. jannaschii* and *E. coli* PyrB and PyrI gene products. The percent of the large hydrophobic residue Ile and the charged residues Lys and Glu are all substantially higher in the PyrB and PyrI gene products of *M. jannaschii* as compared with the corresponding *E. coli* proteins. There is also a large decrease in the percent of Ala residues. A pairwise alignment of the deduced amino acid sequences between the two species indicated that the increased number of Ile residues found in the *M. jannaschii* gene products occurred primarily within the hydrophobic core as deduced from the homology model. Overall, there is a 7 and 6% increase in the number of hydrophobic residues in the *M. jannaschii* PyrB and PyrI gene products, respectively, as compared with the corresponding *E. coli* proteins. The increased incidence of charged residues, Lys and Glu, in the *M. jannaschii* gene products occurred primarily within the hydrophobic core as deduced from the homology model. The thermostability of the *M. jannaschii* PyrB and PyrI gene products may be partially attributed to these substitutions. Other factors may include the decreased number of Trp, Gln, and Cys residues and the increased number of proline and bulkier hydrophobic residues.

**Steady-state Kinetics of the Mj-PyrB Gene Product**—A steady-state kinetic analysis was performed on the purified Mj-catalytic trimer. Initial characterization of the Mj-catalytic trimer was carried out at 25 °C. At this temperature, the Mj-catalytic trimer exhibited Michaelis-Menten kinetics with a maximal velocity 4.6-fold lower and a *K_m* value of aspartate 5.8-fold higher than the *E. coli* catalytic trimer (see Table II). The temperature dependence of the Mj-catalytic trimer enzymatic activity was also investigated. At all temperatures tested, the aspartate saturation curves were hyperbolic. The *E. coli* catalytic trimer demonstrated a significantly higher max-

| Temperature | *M. jannaschii* | *E. coli* |
|-------------|-----------------|----------|
| °C          | Maximal velocity | *K_m*    | Maximal velocity | *K_m*    |
|             | units/mg        | mu       | units/mg        | mu       |
| Aspartate saturation<sup>b</sup> |            |          | Aspartate saturation<sup>b</sup> |            |          |
| 5           | ND              | ND       | 138 ± 5.0       | 1.8 ± 0.08 |
| 15          | 62 ± 3.3        | 40.4 ± 5.0 | 345 ± 33        | 3.1 ± 0.07 |
| 25          | 103 ± 5.0       | 34.6 ± 2.0 | 473 ± 48        | 6.0 ± 0.5 |
| 35          | ND              | ND       | 1013 ± 107      | 20.9 ± 2.8 |
| 37          | 283 ± 38.3      | 18.2 ± 1.0 | ND             | ND       |
| 45          | ND              | ND       | 1337 ± 273      | 39.1 ± 8.4 |
| 55          | 735 ± 105       | 10.9 ± 7.0 | ND             | ND       |
| Carbamoyl phosphate saturation<sup>c</sup> |            |          | Carbamoyl phosphate saturation<sup>c</sup> |            |          |
| 25          | 97 ± 16.7       | 88 ± 1.0 | 473<sup>f</sup> | ND       |
| 37          | 278 ± 46.7      | 162 ± 22 | 20<sup>f</sup> | ND       |

<sup>a</sup> The maximal velocity represents the maximal observed specific activity from the aspartate saturation curve.

<sup>b</sup> These data were determined from aspartate saturation curves. Colorimetric assays were performed in 0.05 M Tris acetate buffer, pH 8.3, at saturating concentrations of carbamoyl phosphate (4.8 mM).

<sup>c</sup> The kinetic parameters at these temperatures were not determined.

<sup>d</sup> Average deviation of at least three determinations.

<sup>e</sup> These data were determined from the carbamoyl phosphate saturation curves. Colorimetric assays were performed in 0.05 M Tris acetate buffer, pH 8.3, and saturating concentrations of aspartate corresponding to four times the half-saturation of aspartate.

<sup>f</sup> Data taken from Stebbins et al. (46).
ormal velocity as compared with that of the Mj-catalytic trimer at all temperatures tested.

Based on the maximal observed specific activities, the activation energies of the transcarbamoylase reaction was calculated to be 9.4 $\pm$ 0.5 and 8.6 $\pm$ 0.5 kcal/mol for the Mj-catalytic trimer and E. coli catalytic trimer, respectively (Fig. 7). The near-equivalence of the two activation energies implies that the rate-limiting step for the Mj-catalytic trimer and the E. coli catalytic trimer are likely the same.

Whereas both M. jannaschii and E. coli catalytic trimers demonstrate catalytic conservation and increased maximal velocity as a function of temperature, binding of substrate to the enzymes follows opposite trends. The $K_m$ value of aspartate for the Mj-catalytic trimer decreases as a function of temperature and, in contrast, the $K_m$ value of aspartate for the E. coli catalytic trimer increases as a function of temperature. Although the activation energies are nearly equivalent for both enzymes, the differences found between $K_m$ of aspartate and maximal velocity may have evolved independently of the catalytic mechanism in order to optimize enzymatic function in the cellular environs of each organism.

In this work we have compared the aspartate transcarbamoylases from E. coli and M. jannaschii. These two organisms are evolutionarily distant, yet their aspartate transcarbamoylases are remarkably similar. Further study of the M. jannaschii enzyme should provide insight into the means of its catalytic mechanism in order to optimize enzymatic function in the cellular environs of each organism.

Acknowledgments—We thank H. Meekins and Dr. M. F. Roberts for growing the M. jannaschii cells.

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