J. Biochem. 115, 309-317 (1994)

Aspartate Aminotransferase from a Thermophilic Formate-Utilizing Methanogen, *Methanobacterium thermoformicicum* Strain SF-4: Relation to Serine and Phosphoserine Aminotransferases, but Not to the Aspartate Aminotransferase Family

Toshio Tanaka,*[,1] Satoshi Yamamoto,* Tatsuki Moriya,* Makoto Taniguchi,* Hideyuki Hayashi, Hiroyuki Kagamiyama,** and Susumu Oi***

**Department of Biology, Faculty of Science, Osaka City University, Sumiyoshi-ku, Osaka 558; and **Department of Biochemistry, Osaka Medical College, Takatsuki, Osaka 569*

Received for publication, October 5, 1993

The primary structure of the aspartate aminotransferase (AspAT) of an archaebacterium, *Methanobacterium thermoformicicum* strain SF-4, has been determined by cloning and sequencing of the gene for the enzyme. The gene had a consensus promoter and a ribosome binding sequence of methanogens in the 5′ untranslated region, followed by an open reading frame starting with ATG and terminating with TGA. The deduced amino acid sequence was identical with the partial amino acid sequences of the enzyme including the N-terminal sequence, and the deduced molecular weight of 41,684 was virtually identical to that reported earlier for this enzyme [Tanaka, T., Yamamoto, S., Taniguchi, M., Hayashi, H., Kuramitsu, S., Kagamiyama, H., & Oi, S. (1992) *J. Biochem.* 112, 811-815]. The gene was expressed in *Escherichia coli* by inserting it into an expression vector just downstream of the *lacZ* promoter, and this verified that the cloned gene really encodes the *Methanobacterium* AspAT. The primary structure of the *Methanobacterium* AspAT showed extremely low homology, 5%, with AspATs of eubacteria, eukaryotes, and a thermoacidophilic archaebacterium, *Sulfolobus solfataricus*. On the other hand, the *Methanobacterium* AspAT showed remarkable amino acid sequence homology, 31.5%, with rat serine:pyruvate aminotransferase and, 13.5%, with *E. coli* phosphoserine aminotransferase. Thus, the *Methanobacterium* AspAT apparently belongs to subgroup IV of the aminotransferases [Mehta, P.K., Hale, T.I., & Christen, P. (1993) *Eur. J. Biochem.* 214, 549-561], but not to subgroup I, in which all the AspATs known so far are included.

Key words: aminotransferase, archaebacterium, aspartate aminotransferase, *Methanobacterium*, methanogen.

Aspartate aminotransferase (AspAT; L-aspartate:2-oxoglutarate aminotransferase) [EC 2.6.1.1] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme, and catalyzes reversible amino group transfer reactions between dicarboxylic amino and oxo acids (*1*). In eukaryote cells, AspAT exists in both the cytosol and mitochondria as two genetically distinct isoenzymes, and the isoenzymes show about 40% homology in the amino acid sequence with each other (*1*). In *Escherichia coli*, and in other bacteria, only one form of AspAT is found. The *E. coli* enzyme shows the same extent of sequence homology (each about 40%) with the cytosolic and mitochondrial isoenzymes (*2*). X-ray crystallographic analyses of pig and chicken isoenzymes, and *E. coli* enzyme showed that these enzymes share a common three-dimensional structure (*3-5*). The active sites of these enzymes are organized with residues which are strictly conserved in the amino acid sequences of the enzyme proteins. The role of these active site residues has been extensively analyzed using X-ray crystallographic and site-directed mutagenesis techniques in combination with spectroscopic and kinetic studies (*6*). Recently, the amino acid sequences of thermostable AspATs from an archaebacterium, *Sulfolobus solfataricus* (*7*), and a eubacterium, *Bacillus* species (*8*), were elucidated. These two enzymes exhibit only 13-14% overall similarity with the eukaryote and *E. coli* enzymes, but show 34% homology with each other. The active site residues identified in the eukaryote and *E. coli* enzymes are conserved in the amino acid sequences of the two thermophilic enzymes (*8*). Therefore, it was suggested that the AspATs of *Sulfolobus*, eubacteria and eukaryotes evolved from a common ancestral protein, and belong to subgroup I of the classification of Mehta *et al.* (*9*). However, only one example of an archaebacterial AspAT, *i.e.*, that from a sulfur-metabolizing bacterium, *S. solfataricus*, is known so far, and thus it is of great interest to characterize AspATs in archaebacteria other than sulfur-metabolizing bacteria, and to elucidate their structures.

The archaebacteria consist of three distinct groups; methanogens, halophiles, and sulfur-metabolizing bacteria (*10*). We previously isolated AspATs from methanogens,

[1] To whom correspondence should be addressed.

Abbreviations: AspAT, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate.

Methanobacterium thermoformicicum and *Methanobacterium thermoautotrophicum*, and showed that they are homotetramers, which has never been reported for AspATs from other organisms (*11, 12*). The enzymes exhibited pH-independent absorption maxima at 415 and 326 nm, whereas AspATs from other sources, except for *Sulfolobus*, showed pH-dependent spectral transitions (*7, 12*). These observations suggest that the *Methanobacterium* AspAT has structurally unique properties which distinguish it from AspATs from other organisms. In the present paper, we present structural evidence that the AspAT from *M. thermoformicicum* SF-4 is distinct from other AspATs, and that the enzyme is closely related with subgroup IV aminotransferases, *i.e.*, serine and phosphoserine aminotransferases.

MATERIALS AND METHODS

Materials—All restriction endonucleases, T4 polynucleotide kinase, the DNA ligation kit, and plasmids pUC18, pUC19, M13mp18, M13mp19, and pTV119N were purchased from Takara Shuzo, Kyoto. Bovine pancreatic ribonuclease was from Pharmacia. Lysyl endopeptidase (*Achromobacter* proteinase I) was a product of Wako Pure Chemical, Osaka. Hybond-N^+ was a product of Amersham, Buckinghamshire, U.K. *E. coli* DH5α, JM103, and BL21 were from Takara. *E. coli* TY103 (*aspC, tyrB*) was obtained in one of our laboratories (*13*). Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were from Sigma Chemical, St. Louis, MO, U.S.A. [γ-^{32}P]ATP (5,000 Ci/mmol) was a product of Du Pont-New England Nuclear, Boston, MA, U.S.A. All other chemicals were of the highest grade commercially available.

Cell Growth and Isolation of DNA—M. thermoformicicum strain SF-4 (DSM 6457) was grown in a synthetic medium as described previously (*14*). Cells were harvested by centrifugation and kept as a suspension in 100 mM Tris-HCl (pH 7.5) at −80°C until use. The bacterial cell lysate was prepared using a pseudomurein endopeptidase from *Methanobacterium wolfei* (DSM 2970), as described below. All procedures were performed in an anaerobic glove box (anaerobic system model 1024; Forma Scientific) because of the severe O_2-sensitivity of the endopeptidase. The frozen suspension of bacterial cells (3 g, wet weight) was thawed and the cells were anaerobically collected by centrifugation using a Nalgen centrifuge tube. The cell pellet was suspended in 30 ml of lysis buffer, which comprised 50 mM potassium phosphate, pH 7.5, 1 M sucrose, 100 mM EDTA, and 250 μl of 2-mercaptoethanol, and then the suspension was centrifuged. The washed cells were resuspended in 30 ml of lysis buffer in a glass vial and then mixed with 12 ml of *M. wolfei* autolysate (*15*), which exhibited no detectable amount of DNA on agarose gel electrophoresis analysis. The vial was sealed with a butyl rubber stopper and an aluminum cap, and then kept at 60°C for 12 h outside the glove box. After the mixture had cooled to room temperature, 25% (w/v) SDS was added to the final concentration of 1% (w/v). Incubation was continued for 10 min for completion of cell lysis. Chromosomal DNA was isolated by the method of Saito and Miura (*16*).

Partial Amino Acid Sequence Analysis and Design of the Oligonucleotide Probe—The purified AspAT (150 μg) from *M. thermoformicicum* strain SF-4 was dissolved in 100 μl of 50 mM Tris-HCl (pH 9.0) and then incubated with lysyl endopeptidase (15 pmol) in 3 μl of distilled water at 35°C for 6 h. The peptide fragments generated were separated by HPLC on a Cosmosil $5C_{18}$ reversed phase column (4.6× 250 mm). Elution was performed with a linear gradient of CH_3CN (0–80%) in 0.1% trifluoroacetic acid at the rate of 1 ml per min at room temperature, and peptide peaks were monitored by measuring the absorbance at 210 nm. The peptide fragments recovered were purified by rechromatography under the same conditions. The major peptide fragments designated as P-14, P-18, P-20, P-21, P-26, P-29, P-31, and P-34 were subjected to amino acid sequence analyses. Their sequences were determined by automated Edman degradation using an Applied Biosystems 470A protein sequencer. In a separate experiment, the *Methanobacterium* AspAT protein was directly analyzed with the protein sequencer, 10 amino acid residues from the N-terminus being determined. A mixed 17-mer oligonucleotide, 5′>GT(AGTC)AT(ATC)ATGGA(AG)GA(AG)GG>3′, corresponding to the sequence of Val-Ile-Met-Glu-Glu-Gly in peptide P-18, was synthesized with an Applied Biosystems 381A DNA synthesizer. The oligonucleotide was 5′-end labeled with [γ-^{32}P]ATP by T4 oligonucleotide kinase (*17*), and then used as a hybridization probe without further purification.

Analysis of the Genomic DNA by Southern Hybridization—Genomic DNA (3.9 μg) from strain SF-4 was completely digested with several restriction endonucleases and then fractionated by agarose gel electrophoresis on 0.8% gel in 40 mM Tris-acetate (pH 8.0)-1 mM EDTA buffer. The DNA fragments in the gel were then transferred to a Hybond-N^+ nylon membrane (80×120 mm) according to the method of Southern (*17, 18*). The DNA-transferred membrane was prehybridized in a 3 ml prehybridization solution for 1 h at 65°C, and then hybridized in the presence of 0.3 pmol (3×10^6 cpm) of the oligonucleotide probe at 44°C overnight as outlined by Maniatis *et al.* (*17*). After the hybridization, the membrane was washed with 4×SSC containing 0.1% SDS for 3 h at 44°C, with several changes of the washing solution. The membrane was then dried and subjected to autoradiography.

Construction of a Genomic DNA Library and Screening—A fragment of about 3.4-kbp *Eco*RI digests showed hybridization with the probe (see "RESULTS AND DISCUSSION"). *Eco*RI-digested *Methanobacterium* genomic DNA (200 μg) was electrophoresed as described above, and the DNA bands of about 3.4 kbp length were excised from the gel and eluted by electrophoresis. The recovered DNA fragments (650 ng) were ligated with *Eco*RI-digested and bacterial alkaline phosphatase-treated pUC18 (100 ng), and then the ligated solution was used to transform *E. coli* DH5α. The transformed cells were then plated on 1.5% agar plates of LB medium supplemented with 50 μg/ml ampicillin to give almost 400 ampicillin-resistant colonies per plate. About 4,000 transformants were subjected to the colony hybridization test with the above oligonucleotide probe according to standard procedures (*17*), and 5 clones were found to be positive. The positive clones were isolated, and a repeated hybridization test showed that 2 of the 5 clones were clearly positive. These two clones were subjected to further analysis.

Nucleotide Sequence Analysis of the Cloned DNA—The

insert DNA of pSYE5 was digested with appropriate restriction endonucleases and the generated DNA fragments were separated on agarose gels by electrophoresis and recovered. The fragments were subcloned into M13mp18 or M13mp19, and single-stranded DNA was obtained from each subclone. Nucleotide sequence analyses were performed by the dideoxy chain termination method using an Applied Biosystems Dye Deoxy Terminator Taq sequencing kit and a model 370A automated DNA sequencer.

Construction of an Expression Vector—A *Methanobacterium* AspAT expression vector was constructed by inserting the open reading frame of the gene just downstream of the *lacZ* promoter of plasmid pTV119N. A 2.4-kbp *Sal*I-*Kpn*I fragment covering the coding region of AspAT was excised from pSYE5, and subcloned into the corresponding restriction sites of pTV119N. The resulting plasmid was used to transform *E. coli* JM103, and single-stranded DNA as a template for mutagenesis was recovered by cultivation in the presence of a helper phage, M13KO7. A *Nco*I recognition sequence was newly introduced at the initiation codon, ATG, of the gene by site-directed mutagenesis using a 25-mer oligonucleotide (5′>GGTTTTTTGCCATGGATGAAACAC>3′, mismatches are underlined), synthesized similarly to as described above, and an oligonucleotide-directed *in vitro* mutagenesis system, Version 2.1 (Amersham, U.K.). The resulting mutant plasmid was excised with *Nco*I and then religated to give plasmid pTM115N. In this plasmid, a 1.6-kbp fragment of the gene for *Methanobacterium* AspAT exists at the *Nco*I-*Kpn*I site of pTV119N, and the initiation codon, ATG, is located just downstream of the Shine-Dalgarno sequence of the original *lacZ* gene. The nucleotide sequence around the translation initiation site and the entire coding region was confirmed by dideoxy sequencing as described above.

Assaying of AspAT Expressed in E. coli Cells—Cells of various *E. coli* strains were grown in 100 ml of LB medium supplemented with ampicillin (50 μg/ml) and 0.4 mM IPTG overnight at 37°C with vigorous shaking. Cells were harvested by centrifugation and suspended in 3 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 100 μM PLP, and then disrupted by sonication. The supernatant obtained on centrifugation was used for the assaying of AspAT according to the method described previously (*11*). The protein content was measured by the method of Bradford (*19*) using bovine serum albumin as a standard. PAGE was performed under non-denaturing conditions (*12*), AspAT being localized by activity staining of the gel according to the method of Yagi *et al.* (*20*).

Substrate Specificity—The activity of the enzyme as to transamination of the alanine-2-oxoglutarate pair was assayed by measuring pyruvate as a phenylhydrazone (*11, 12*). The activity as to the phosphoserine-2-oxoglutarate pair was assayed by measuring the product, glutamate, with a glutamate dehydrogenase-NAD^+ system (*11*). The activities as to the aspartate-glyoxylate and alanine-glyoxylate pairs were assayed by measuring pyruvate with a lactate dehydrogenase-NADH system (*21*). All the transamination reactions were performed under the same conditions as those used for assaying AspAT activity (*11, 12*): 41.7 mM of an amino donor, 4.17 mM of an amino acceptor, 10 μM PLP, at 60°C. The reactions were stopped by boiling the mixtures at 100°C for 10 min, and the products were measured as described above.

RESULTS AND DISCUSSION

Cloning of the AspAT Gene—The genomic DNA of *M. thermoformicicum* SF-4 was digested with several restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sse*8387I, and *Xba*I), and then electrophoresed on an agarose gel. Southern analysis showed that only one restriction fragment hybridized to the probe, irrespective of the restriction enzyme used to digest the DNA (data not shown). This indicates that there is only one copy of the AspAT gene in the genome of *M. thermoformicicum* SF-4. Among various restriction endonucleases, *Eco*RI was found to give enough fragments (3.4 kbp) to cover the coding region for a 43 k subunit of *Methanobacterium* AspAT. Therefore, this *Eco*RI fragment was cloned as described under "MATERIALS AND METHODS." Two clones were shown to be positive upon hybridization with the probe. The recombinant plasmids isolated from the two clones carried a 3.4-kbp insert in the *Eco*RI restriction site of pUC18. Restriction mapping of the insert DNA (Fig. 1) showed that the two clones were identical. Therefore one of the clones, named pSYE5, was subjected to DNA sequence analysis.

Nucleotide Sequence of the AspAT Gene and the Deduced Amino Acid Sequence of AspAT—Analyses of the restriction fragments of the 3.4-kbp insert DNA of plasmid pSYE5 by Southern hybridization showed that the 2.4-kbp *Sal*I-*Eco*RI fragment hybridized to the probe. This fragment was therefore subjected to nucleotide sequence

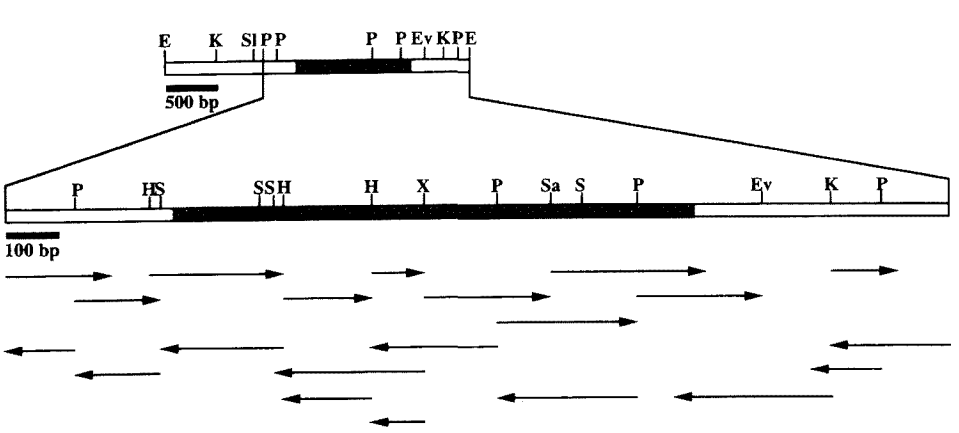

Fig. 1. **Restriction map of the *M. thermoformicicum* AspAT gene and flanking regions.** The upper part of the figure shows the 3.4 kbp *Eco*RI insert of pSYE5. The lower part shows the detailed restriction map of the region from the first *Pst*I site to the *Eco*RI site. The DNA sequencing strategy is indicated by arrows. Hatched areas indicate the open reading frame of *Methanobacterium* AspAT. Abbreviations: E, *Eco*RI; Ev, *Eco*RV; H, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sau*3A; Sa, *Sac*I; Sl, *Sal*I; X, *Xho*I.

analysis. Most of the sequence of the fragment was determined according to the strategy shown in Fig. 1. The nucleotide sequence, together with the deduced amino acid sequence and proposed promoter structure is shown in Fig. 2. The N-terminal 10 residue sequence of the *Methanobacterium* AspAT was found between the second *Pst*I site and

```
                                                                CTGCAGATACCACGGTAAGGTTCT -361
CATCCAGTCCCCTGAACCTCTCAGCATGCTCCTTCAGGGATGGTCCTGCTCCGAAGACTATGAAGTTCCTTGATGGGAGTTCCAGTCCAT -271
GGAGGTCGATGCTCCCGTGTTCTTCAAGGAACCCTCTCAGGTACTCTGCAGCCTCCTCATCGGCGGCCCTGTCAAATCCAAAGTCTTCAA -181
GTATCCCTGCATACCACCTTAACCACACTTCAAGTTCCATGTGATAAATTTGTAGGCCTTAATATATCATGCTTTTGTTCCAGAGGGAC  -91
GCCCTTCTGGTGGGTGTAAATATTTTTAAAGGTACAGATTAAATAATAGTAACAGCTCTAAAACGCACTTTAATCTGGAGGTTTTTTGGA   -1

ATGGATGAAACACTGCTCATGATACCTGGCCCTACAAGGGTGGCCCAGAGAGTCCTTAAGGCAATGTCAGAGAACATTGTTAACCACAGG   90
MetAspGluThrLeuLeuMetIleProGlyProThrArgValAlaGlnArgValLeuLysAlaMetSerGluAsnIleValAsnHisArg   30
                                                                                    P-26

AGCGCATTATTTGGTAAGATCCTCTCTGAAACCAGTGAGATGATGTCCGATGTCTTCAGGACAAACAATAAGTCTTACCTTTTAACAGGC  180
SerAlaLeuPheGlyLysIleLeuSerGluThrSerGluMetMetSerAspValPheArgThrAsnAsnLysSerTyrLeuLeuThrGly   60
                                                    P-31

TCGGGGACAGCCGCAATGGAAGCTGCAATCGCAAATATAATTGAGCCGGGTGATAAGGTCCTCAATGTAGTTGGGGGCAAGTTCGGTCAG  270
SerGlyThrAlaAlaMetGluAlaAlaIleAlaAsnIleIleGluProGlyAspLysValLeuAsnValValGlyGlyLysPheGlyGln   90

AGGTTCAGCCAGATCGTTGAGGCCTTCGGCGGCGAGTCCATAAGGATCGATGTTGAATGGGGTAAAGCCGTTAACCCGGATGAAATCGGC  360
ArgPheSerGlnIleValGluAlaPheGlyGlyGluSerIleArgIleAspValGluTrpGlyLysAlaValAsnProAspGluIleGly  120
                                                                                    P-29

TACGCACTCGAAGAGAACGATGATATAAAGGCAGTTACAGTCGTCCACAATGAAACATCCACCGGTGTTGCAAACCCTATAAAGGAGATA  450
TyrAlaLeuGluGluAsnAspAspIleLysAlaValThrValValHisAsnGluThrSerThrGlyValAlaAsnProIleLysGluIle  150
                                                    P-20

GGGAAGATAATGTCAGATTATGACGCCCTCTACGTTGTTGATACTGTTTCATCCCTGGGCGGGGATGAGGTTGACGTTGATGGTTATGGT  540
GlyLysIleMetSerAspTyrAspAlaLeuTyrValValAspThrValSerSerLeuGlyGlyAspGluValAspValAspGlyTyrGly  180

ATAGACATATGTGTAACCGGCTCCCAGAAGTGCCTTGCAGCACCACCGGGTATGGCGGCCATAACCCTGAGTGACGATGCATGGAGCGCC  630
IleAspIleCysValThrGlySerGlnLysCysLeuAlaAlaProProGlyMetAlaAlaIleThrLeuSerAspAspAlaTrpSerAla  210

GTTGAAGGAGTCAGCAACTCGAGGACATATTACCTGAACCTTAAAAAAGTACAGGAAGAGCGGCGACGCTGAACCCCCTGAAACACCATAC  720
ValGluGlyValSerAsnSerArgThrTyrTyrLeuAsnLeuLysLysTyrArgLysSerGlyAspAlaGluProProGluThrProTyr  240

ACCCCTGCGGTTTCACTCATCTACGCAATGCACGAGCCCCTTAAGGTTATAATGGAGGAGGGCCTTAACAACAGGATAAAGAGGCATAAA  810
ThrProAlaValSerLeuIleTyrAlaMetHisGluProLeuLysValIleMetGluGluGlyLeuAsnAsnArgIleLysArgHisLys  270
                                                    P-18

CTTGCTGCAGAGGCAACAAGGAATGCTATAAAGGCACTTGGCCTTGAACTCTTCCCTGATGAGTCTGTTTCATCAACCACGGTGACCGCG  900
LeuAlaAlaGluAlaThrArgAsnAlaIleLysAlaLeuGlyLeuGluLeuPheProAspGluSerValSerSerThrThrValThrAla  300
          P-14                                                      P-34

GTAAGACTCCCTGAAGGGGTTACGGACGGGGAGCTCAGGGGTACAATGAGGAACAAGTACCATGTTGAGCTTGCAGGTGGACAGGACCAT  990
ValArgLeuProGluGlyValThrArgAspGlyGluLeuArgGlyThrMetArgAsnLysTyrHisValGluLeuAlaGlyGlyGlnAspHis  330
                                                                                    P-21

CTGAAGGGCAAGATCTTCAGGATAGGGCATATGGGTAACATAACCCACAGGGAACTGATAACCACCATTTCAGGTCTGGAGATGACCCTG 1080
LeuLysGlyLysIlePheArgIleGlyHisMetGlyAsnIleThrHisArgGluLeuIleThrThrIleSerGlyLeuGluMetThrLeu  360

AGGGAACTTGGATTCGAGGTTGAGATGGGCGAGGCCGTGGCTGCAGTTGCAGACACCTACCTTCCTGAGAATCTCTGAACCACACCCCCA 1170
ArgGluLeuGlyPheGluValGluMetGlyGluAlaValAlaAlaValAlaAspThrTyrLeuProGluAsnLeu***              385

TTTACTTTTTATATGGCAGTAATCTAACTGGAGGTATTATTTTAGTTCCGTCAGCAGCGGGATTTTTCCTGATACCTTTCTCAGGTGTAT 1260
TTTAGTTCCATTTCAGTAGCATTCCATGATAACCCTTTTCTCAATAAAAGCGGCTTTATAAATCCTTGGAGCAGAGGCTTATATCCCTGT 1350
ATCTTCATGGAGTCCGGTGGTCGGGTAGGGTATATCCTCTTCACCAACCTTGATATCCAGAAGGAATGGTTCTCTGGATGCAAGGGCCAT 1440
TTCCAGGGAATCTGAGAGGTCATCATAGCTTTCAACACCCACGGCACCTATACCATAGGCTCCTGCAAGTTTCACGAAGTCAGGGTTCTT 1530
CAGTTCAACACCGTAACTTTCACCGTACTTCATCTCCTGCCACTGCCTTATGACATCCAGCCGGCTGTTGTTCAGGATGCACATTGTAAC 1620
AGGAAGTCCAAGTTCAGCCGCGGTACCGAGTTCCTGCATGGTCATCTGGAACCCGCCATCACCTGCTATGAGAACCACATCCTCATGGGG 1710
CTCGGCAAGGCTCGCCCCCACCGCAGCAGGAAGACCATAACCCATGGGTCCGAATCCACCTGAGAATATAAGTGAGCGCTCCCTGAGGAC 1800
CTTCCTGCAGAGGGTCACCCAGGTTGTGTGGCTGCCAGCATCATTAACTATTATGGCATCGGGGGCTGCTTCAAGGATTCCCCTTACAGC 1890
AAGGGAGGTCCTGAATTC                                                                          1908
```

Fig. 2. **Nucleotide sequence of the gene encoding *M. thermoformicicum* SF-4 AspAT and the deduced amino acid sequence.** The numbering of nucleotides is relative to the first nucleotide of the coding region. The sequences identical to those determined for the N-terminus and the lysyl endopeptidase digest are underlined. For peptides P-20 and P-34, the entire sequences were not obtained by Edman degradation. The proposed unanalyzed portions of these peptides are denoted by dashed underlines. The potential promoter sequences (AAATTT and TTAATATA) and ribosome-binding sequence (GGAGG) are boxed.

TABLE I. **Activity of the expression product of *Methanobacterium* AspAT gene and its thermostability.** *E. coli* BL21 and transformed *E. coli* TY103 cells were cultured in 100 ml of LB medium overnight. Cells were harvested by centrifugation, disrupted by sonication in 3 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 100 μM PLP, and again centrifuged. The supernatants were analyzed as to AspAT activity and protein concentration as described under "MATERIALS AND METHODS." Heat treatment was performed at 60°C for 90 min. n.d., activity was not detected. From the detection limit for measuring activity in our system, it was estimated to be lower than 0.001 U/mg.

Bacterial strain/plasmid	Activity (μmol·min^{-1}·mg^{-1})
BL21	0.29
BL21, heat treated	0.007
TY103/pTV119N	n.d.
TY103/pTV119N, heat treated	n.d.
TY103/pTM115N	0.045
TY103/pTM115N, heat treated	0.21

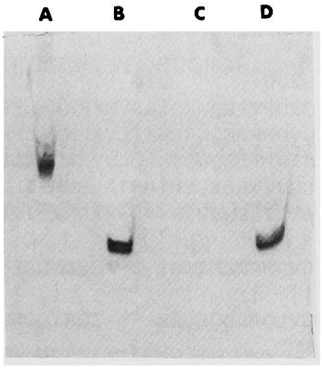

Fig. 3. **Analysis of the AspAT gene product by non-denaturing PAGE.** Lane A: The cell free extract of *E. coli* BL21 (18 μg of protein) without heat treatment. Lane B: The cell free extract of *E. coli* TY103 transformed with pTM115N (30 μg of protein) after heat treatment at 60°C for 90 min. Lane C: The cell free extract of *E. coli* TY103 transformed with pTV119N (30 μg of protein) after heat treatment at 60°C for 90 min. Lane D: Purified *M. thermoformicicum* SF-4 AspAT (0.3 μg of protein). Electrophoresis was performed on a 7.5% gel, followed by detection of AspAT activity by incubation of the gel with a mixture of 50 mM L-cysteine sulfinate, 5 mM 2-oxoglutarate, and 15 μM PLP in 100 mM Tris-HCl (pH 7.5) at 60°C, as described by Yagi *et al.* (*20*).

the first *Hinc*II site of the insert DNA fragment. From the putative initiation codon, ATG, an open reading frame of 1,155 bp, which is terminated by a termination codon, TGA, was found. The encoded polypeptide consisted of 385 amino acid residues, the molecular weight being predicted to be 41,684. This was consistent with the value of 43 k for the subunit of this enzyme, which was determined on SDS-PAGE (*12*). All the sequences of the *Achromobacter* proteinase I peptides which were isolated and sequenced were found in the open reading frame (Fig. 2).

The G+C content of the open reading frame was 50.1%, which is similar to the value (50.7%) obtained for the genomic DNA of strain SF-4 (*14*). The nucleotide sequence of the region upstream of the putative initiation codon was examined for promoter and ribosome-binding structures. In methanogens, various promoter sequences have been identified for genes encoding polypeptides (*22-25*). The promoter element usually consists of two different boxes which are separated by an intergenic sequence (*23*). In

TABLE II. **Amino donor and acceptor specificity of *Methanobacterium* AspAT.** The reactions catalyzed by AspAT purified from *M. thermoformicicum* SF-4 were performed at 60°C in the presence of 41.7 mM amino donor, 4.17 mM amino acceptor, and 10 μM PLP. The reactions were started by the addition of the enzyme and terminated by boiling the assay mixture at 100°C for 10 min. The products were measured as described in the text. n.d., not determined.

Amino acceptor	Aminotransferase activity (μmol·min^{-1}·mg^{-1}) with amino donor of		
	Aspartate	Alanine	Phosphoserine
2-Oxoglutarate	21.2	0.77	0.65
Glyoxylate	1.89	0.69	n.d.

polypeptide-encoding genes, the consensus sequence of the upstream box, A, is GAANTTTCA, where N is any base, and that of the downstream box, B, is TTTAATATAAA (*23*). The 5′-flanking region of the *Methanobacterium* AspAT structural gene contained the sequences of box A (AAATTT) and box B (TTAATATA), starting at 158- and 144-bp upstream, respectively, of the ATG coding the N-terminal methionine. In most polypeptide-encoding genes of methanogens, the region immediately upstream of the translation initiation codon is known to have complementary sequences for hybridization with the 3′ end sequence of 16S rRNA (3′<UCCUCCACUAGGUCGG<5′) (*23*). The sequence, GGAGGT, which is 10 bases upstream of the ATG, was determined to be a ribosome-binding region. These results strongly suggest that the ATG coding the methionine residue at the N-terminus of the *Methanobacterium* AspAT is the initiation codon, and that the AspAT structural gene has a regulatory element in its 5′-flanking region.

Validation of the AspAT Gene by Expressing the Enzyme Protein—As discussed above, the *Methanobacterium* AspAT gene has a typical methanogen promoter sequence in its 5′-untranslated region, and is not likely to be directly expressed in *E. coli*. Accordingly, *E. coli* TY103 cells transformed with pSYE5 did not show any detectable AspAT activity (data not shown). It has also been shown that the *glnA* gene of *Methanococcus voltae* was not effectively expressed in *E. coli* cells, possibly due to the upstream promoter and ribosome-binding sequences specific to methanogens (*26*). Therefore, in order to express the *Methanobacterium* AspAT in *E. coli*, an expression vector, pTM115N, in which the structural gene was directly placed just downstream of the *lacZ* gene promoter was constructed. Table I shows the result of expression of *Methanobacterium* AspAT in *E. coli* cells. The background AspAT activity in *E. coli* TY103 cells was negligible because of the genetic deletion of both AspAT and aromatic amino acid aminotransferase (*13*). On the other hand, AspAT activity was detected in the crude extract of TY103 cells carrying pTM115N. The activity was stable after heat treatment at 60°C for 90 min in the presence of 100 μM PLP, under which the *E. coli* AspAT lost its activity (see the activity of *E. coli* BL21). This heat treatment resulted in a 5-fold increase in the specific activity due to denaturation of proteins from the host cells. The cell extracts in Table I were analyzed by native PAGE, and AspATs were detected by activity staining (Fig. 3). The active band of TY103 transformed with pTM115N (lane B) showed the same mobility as that from *M. thermoformicicum* strain SF-4

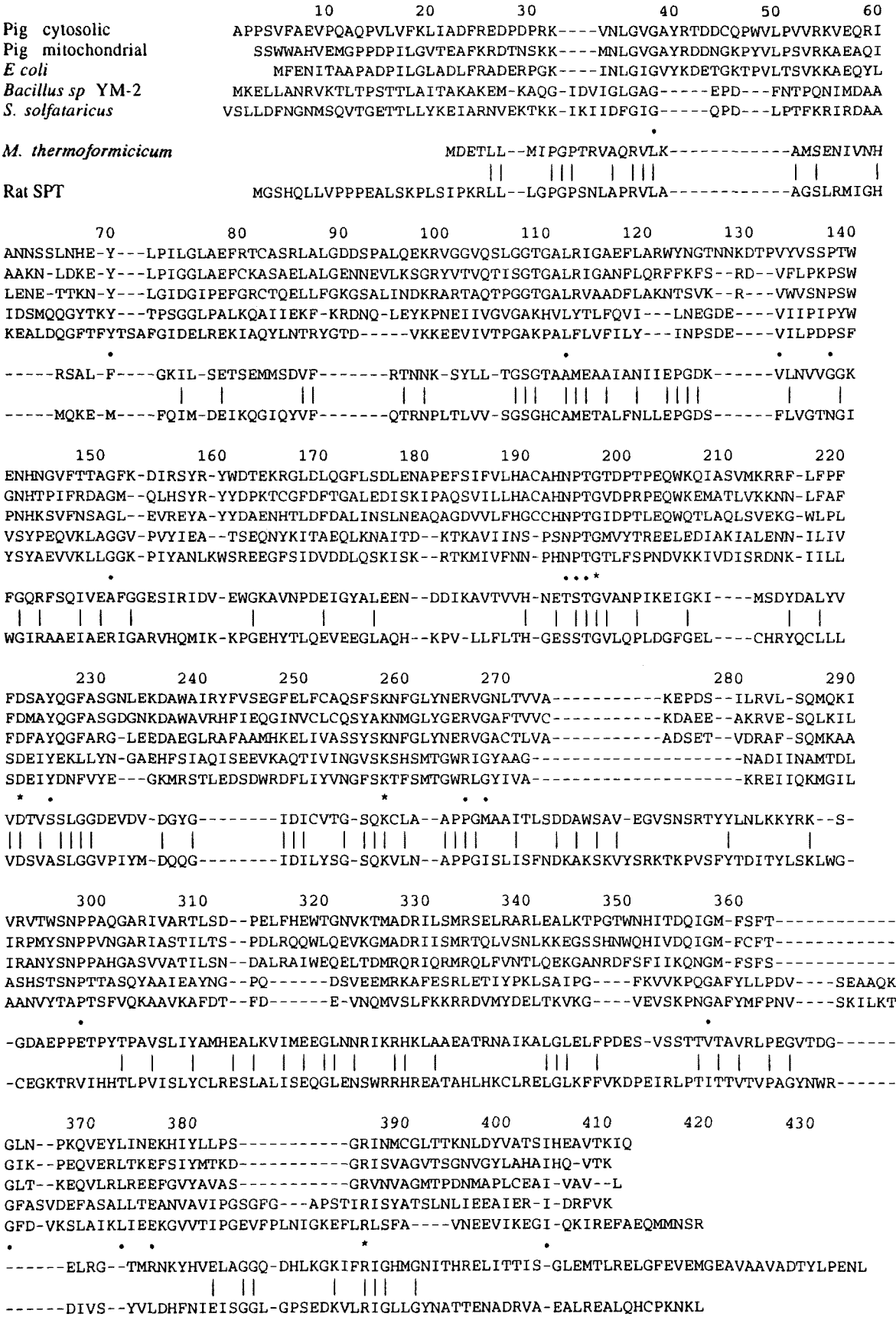
Pig cytosolic
Pig mitochondrial
E coli
Bacillus sp YM-2
S. solfataricus
M. thermoformicicum
Rat SPT

Fig. 4

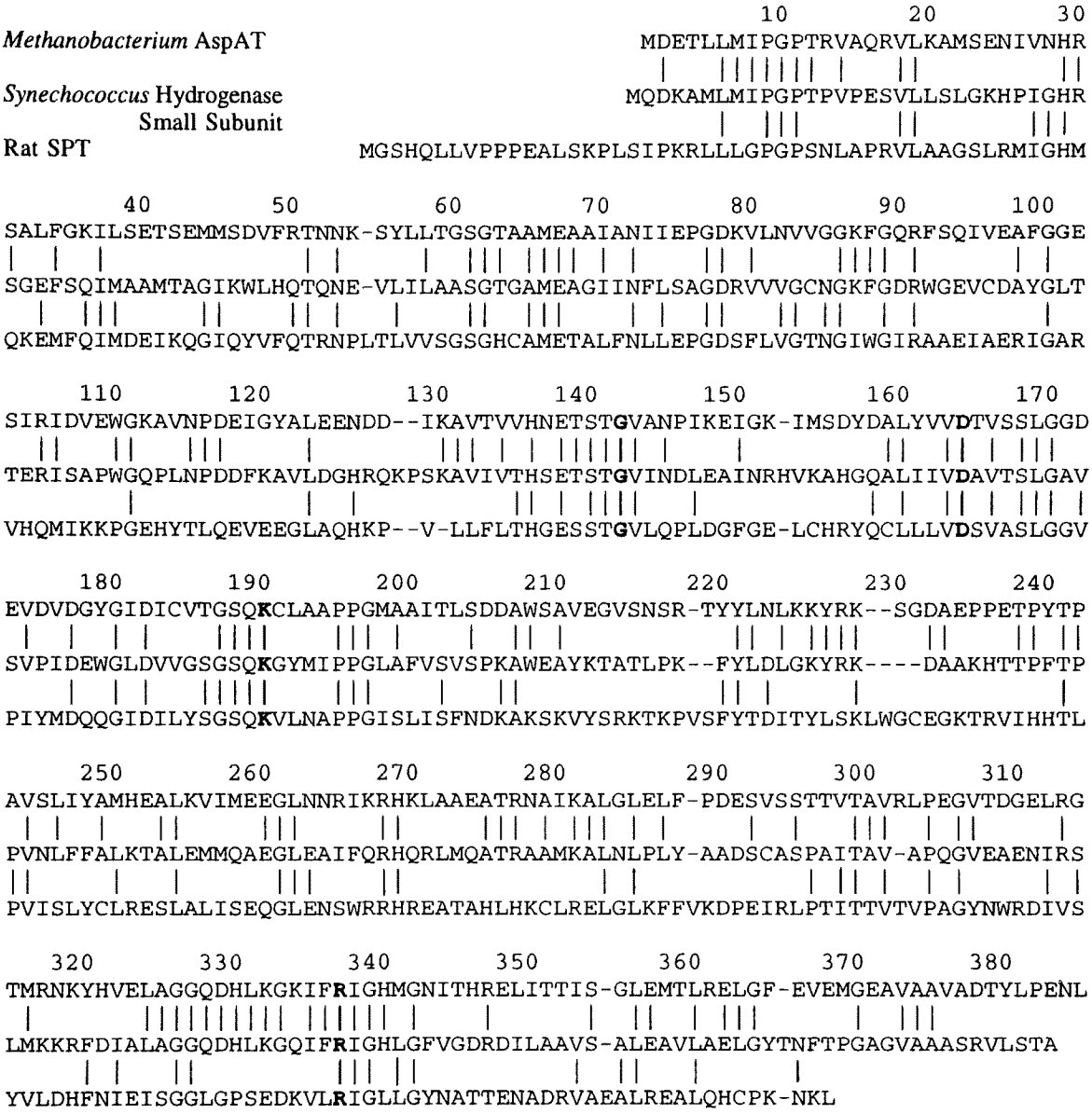

Fig. 5. **Comparison of the amino acid sequences of *Methanobacterium* AspAT, rat serine:pyruvate aminotransferase (*30*), and *Synechococcus* hydrogenase (*38*).** The amino acid residue numbers are those of *Methanobacterium* AspAT. Bars and dashes are used similarly to as in Fig. 4. Bold letters indicate the residues which are conserved throughout the four subgroups.

Fig. 4. **Comparison of the amino acid sequences of AspATs from various sources and rat serine:pyruvate aminotransferase.** The AspAT sequences are those of pig cytosol (*27, 28*), pig mitochondria (*29*), *E. coli* (*2*), *Bacillus* sp. YM-2 (*8*), *S. solfataricus* (*7*), and *M. thermoformicicum* (this work), and are compared to rat serine:pyruvate aminotransferase (*30*). The amino acid residues are numbered according to the sequence of the pig cytosolic enzyme. Dashes indicate deletions introduced to maximize the identities of amino acid residues between the AspATs. The residues which are conserved between *Methanobacterium* AspAT and rat serine: pyruvate aminotransferase are indicated by bars between the two sequences. The residues which are conserved among AspATs except *Methanobacterium* AspAT are denoted by dots (·). The residues which appear to be identical between AspATs including *Methanobacterium* AspAT and subgroup IV aminotransferases are indicated by asterisks (*).

(lane D), which is clearly distinguishable from the band of *E. coli* AspAT with a lower electrophoretic mobility (lane A). These results clearly show that the gene we have isolated is indeed the structural gene for the *M. thermoformicicum* SF-4 AspAT. The expression level is, however, much lower than that expected from the well-designed organization of the promoter and the coding frame in pTM115N. The preparation of a homogeneous AspAT protein requires almost 100-fold purification, even from a heat-treated sample, as estimated from the specific activity of the purified preparation (*12*). Experimental conditions are under investigation to enhance the production of this heterologous AspAT in *E. coli* cells, to facilitate the structural study of this unique enzyme.

Primary Structure of Methanobacterium AspAT—The above results and our previous findings indicate that

Methanobacterium AspAT is a tetrameric protein composed of four identical 42 k subunits. Like other AspATs from several sources, *Methanobacterium* AspAT is a PLP-dependent enzyme which contains one molecule of the cofactor per one subunit (*12*). The absorption spectra of the enzyme suggested the presence of a Schiff base formed between PLP and an amino group of the enzyme (*12*), but attempts to fix the PLP molecule to the enzyme by reduction with sodium borohydride failed. The addition of 10 mM sodium borohydride did not effectively abolish the 415 nm absorption. Further experiments are needed to determine whether PLP is bound to a lysine residue or not, but it is worthwhile to consider the possibility that the microenvironment of the active site of *Methanobacterium* AspAT may make the Schiff base resistant to reduction by borohydride ions.

The primary structure of *Methanobacterium* AspAT was compared to those of AspATs from pig cytosol (*27, 28*), pig mitochondria (*29*), *E. coli* (*2*), *Bacillus* sp. (*8*), and *Sulfolobus solfataricus* (*7*). However, under standard conditions of stringency, *Methanobacterium* AspAT showed no significant sequence homology with these eukaryote, eubacterial, and archaebacterial AspATs. A more general comparison was performed by comparing the sequence of *Methanobacterium* AspAT with those of the proteins in the DNA Data Bank of Japan using DDBJ FASTA. The result showed that *Methanobacterium* AspAT exhibits apparent homology with serine:pyruvate aminotransferase of rat (*30*) and human (*31*), the percentages being 31.5 and 31.8%, respectively. *Methanobacterium* AspAT also showed significant homology, 13.5%, with phosphoserine aminotransferase of *E. coli* (*32*) and rabbit (*33*), which together with serine:pyruvate aminotransferase forms subgroup IV of the aminotransferases (*9*). Clearly, *Methanobacterium* AspAT belongs to subgroup IV, but *not* to subgroup I, in which all other AspATs are included. Subgroups IV and I, however, exhibit faint similarity in several restricted portions, and it is reasonably argued that the two subgroups, and possibly the other two subgroups, might be homologous enzyme groups (*9*). Accordingly, the sequences of *Methanobacterium* AspAT and rat serine: pyruvate aminotransferase were aligned with those of the AspATs of subgroup I (Fig. 4). On this alignment, *Methanobacterium* AspAT showed 4 to 5% homology with subgroup I AspATs. The invariant residues among AspATs, according to the residue numbers of porcine cytosolic AspAT, are Gly197, Asp222, Lys258, and Arg386. These 4 residues are also invariant throughout the 4 subgroup enzymes (*9*). That *Methanobacterium* AspAT indeed has these residues indicates the validity of the sequence alignment in Fig. 4 and in Ref. *9*. Among these invariant residues, Lys258 is the PLP-binding lysine, and Asp222 forms a hydrogen bond/ salt bridge to the protonated pyridine N of PLP (*1-5*). Arg386 acts as the anchoring site for the α-carboxylate group of L-amino acids, and Gly197 is thought to play a key role in the folding of the polypeptide chain at the subunit interface near Arg386 (*1-3, 34*). The conservation of the above 4 residues suggests the resemblance of the spatial structures of the enzymes of subgroups other than I to those of AspATs (*9*). However, there are several residues whose importance has been well documented in AspATs but which are not conserved in the enzymes of the other 3 subgroups. These residues include Tyr70, Asn194, Tyr225, and Arg266, which directly interact with PLP, and are involved in the binding of PLP and modulation of the catalytic function of the enzymes through interaction with PLP (*9*). Possibly in subgroups II, III, and IV, other residues may substitute for these residues. It is of interest to correlate the lack of residues corresponding to Asn194 and Tyr225 in *Methanobacterium* AspAT with its spectroscopic property. *Methanobacterium* AspAT does not show pH-dependent spectral change, which is commonly observed for other AspATs (*1*), but not for the *Sulfolobus* enzyme (*7*). The absorption spectrum shows pH-independent peaks at 415 and 326 nm (*12*), and this indicates the presence of a protonated Schiff base (*1, 35*). Therefore, the pK_a of the imine nitrogen of the Schiff base is considered to be remarkably high in *Methanobacterium* AspAT. In the other AspATs, the pK_a falls in the range of 6.5 to 7.0, and these low pK_a values are at present considered to be brought about by (i) protonation at the pyridine nitrogen of PLP, which is stabilized by the negative charge of Asp222, and (ii) hydrogen bonding to the 3′-O of PLP from Asn194 and Tyr225 (*1*). Site-directed mutagenesis studies on the *E. coli* enzyme showed that Asn194 and Tyr225 lower the pK_a value by 1.7 and 2 units, respectively, and when both of the residues were mutated, the pK_a value increased to more than 11 (*36*). Therefore the pH-independent nature of the spectra of *Methanobacterium* AspAT may be brought about by the lack of hydrogen bonds to the 3′-O of PLP, which are provided by Asn194 and Tyr225 in the other AspATs. Furthermore, in *Methanobacterium* AspAT, the absorption peak at 326 nm is similar in height to that at 415 nm, showing the significant amount of enolimine structure in the PLP-lysine Schiff base. *E. coli* AspAT, in which Asn194 and Tyr225 are replaced by alanine and phenylalanine residues, respectively, gave a similar absorption spectrum to that of *Methanobacterium* AspAT, with increased absorption at 335 nm and decreased absorption at 430 nm (*36*). It is reasonable to consider that the lack of hydrogen bonds to 3′-O of the PLP moiety promotes the protonation at this atom, and thus the PLP-lysine Schiff base prefers the enolimine structure to the ketoenamine structure.

That *Methanobacterium* AspAT structurally belongs to the subgroup IV aminotransferases requires careful re-examination of its substrate specificity (*11*). As reported earlier, when 2-oxoglutarate was used as an amino acceptor, the enzyme showed no activity toward alanine or phenylalanine (*11*). In this study we additionally measured the activities as to the transamination of several pairs of amino donors and acceptors (Table II). The activity of the enzyme as to the transamination of any of these pairs was less than 10% of that in the case of aspartate and 2-oxoglutarate. Therefore the enzyme is highly specific to aspartate and 2-oxoglutarate. This conclusively shows that in *Methanobacterium* the aminotransferase specific to aspartate is a subgroup IV aminotransferase.

It has been shown (*37*) that subgroup IV aminotransferases exhibit homology with the small subunit of cyanobacterium soluble hydrogenases (*38, 39*). Regarding the two aminotransferases of subgroup IV, serine-pyruvate aminotransferase showed a higher homology value (28%) than phosphoserine aminotransferase (6-7%). *Methanobacterium* AspAT showed larger homology values (around 40%) with hydrogenases than serine-pyruvate aminotransferase (Fig. 5). The biological significance of the fact that the small

subunits of soluble hydrogenases, together with other NifS family proteins, are homologous with aminotransferases has been discussed, and the possibility of the NifS proteins functioning as aminotransferases during nitrogen fixation has been postulated (*37*). Apparently, among the aminotransferases, *Methanobacterium* AspAT has the highest homology with the small subunit of hydrogenases, and it is of great interest to investigate the evolutional relationship between *Methanobacterium* aminotransferases and NifS proteins.

The four subgroups of aminotransferases proposed by Mehta *et al.* are thought to have evolved from a common ancestral protein, the divergence having occurred before the emergence of the three biological kingdoms (*9*). The two archaebacterial aminotransferases, *S. solfataricus* AspAT and *Haloferax volcanii* histidinol phosphate aminotransferase, are both subgroup I aminotransferases. *Methanobacterium* AspAT is the first archaebacterial aminotransferase which belongs to a subgroup other than subgroup I. This indicates the validity of Mehta's classification throughout the three kingdoms, and supports the hypothesis of evolutional branching of the subgroups in the ancestral cells. Specialization of the aminotransferases as to substrate specificity and division into structural subgroups are not strictly correlated events, because the present results show that not only a subgroup I enzyme but also a subgroup IV enzyme could acquire specificity toward aspartate. The modes of binding of substrates and analogs to *Methanobacterium* AspAT, which will be revealed on crystallographic analysis of the enzyme, will give us a new type of transamination mechanism for dicarboxylic amino and oxo acids. For this purpose, the construction of an overproduction system for the enzyme in *E. coli* is required, which is now underway in our laboratories.

REFERENCES

1. Christen, P. & Metzler, D.E. (1985) *Transaminases*, John Wiley & Sons, New York
2. Kondo, K., Wakabayashi, S., Yagi, T., & Kagamiyama, H. (1987) *J. Biol. Chem.* **262**, 8648-8657
3. Jansonius, J.N., Eichele, G., Ford, G.C., Picot, D., Thaller, C., & Vincent, M.G. (1985) in *Transaminases* (Christen, P. & Metzler, D.E., eds.) pp. 110-138, John Wiley & Sons, New York
4. Arnone, A., Rogers, P.H., Hyde, C.C., Briley, P.D., Metzler, C.M., & Metzler, D.E. (1985) in *Transaminases* (Christen, P. & Metzler, D.E., eds.) pp. 138-154, John Wiley & Sons, New York
5. Kamitori, S., Okamoto, A., Hirotsu, K., Higuchi, T., Kuramitsu, S., Kagamiyama, H., Matsuura, Y., & Katsube, Y. (1990) *J. Biochem.* **108**, 175-184
6. Fukui, T., Kagamiyama, H., Soda, K., & Wada, H. (1991) *Enzymes Dependent on Pyridoxal Phosphate and Other Carbonyl Compounds as Cofactors*, Pergamon Press, Oxford
7. Cubellis, M.V., Rozzo, C., Nitti, G., Arnone, M.I., Marino, G., & Sannia, G. (1989) *Eur. J. Biochem.* **186**, 375-381
8. Sung, M.-H., Tanizawa, K., Tanaka, H., Kuramitsu, S., Kagamiyama, H., Hirotsu, K., Okamoto, A., Higuchi, T., & Soda, K. (1991) *J. Biol. Chem.* **266**, 2567-2572
9. Mehta, P.K., Hale, T.I., & Christen, P. (1993) *Eur. J. Biochem.* **214**, 549-561
10. Woese, C.R. & Olsen, G.J. (1986) *Syst. Appl. Microbiol.* **7**, 161-177
11. Tanaka, T., Tokuda, T., Tachibana, A., Taniguchi, M., & Oi, S. (1990) *Agric. Biol. Chem.* **54**, 625-631
12. Tanaka, T., Yamamoto, S., Taniguchi, M., Hayashi, H., Kuramitsu, S., Kagamiyama, H., & Oi, S. (1992) *J. Biochem.* **112**, 811-815
13. Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., Hiromi, K., & Kagamiyama, H. (1991) *J. Biol. Chem.* **266**, 6079-6085
14. Yamamoto, K., Tachibana, A., Dhavises, G., Tanaka, T., Taniguchi, M., & Oi, S. (1989) *Agric. Biol. Chem.* **53**, 533-534
15. Kiener, A., König, H., Winter, J., & Leisinger, T. (1987) *J. Bacteriol.* **169**, 1010-1016
16. Saito, H. & Miura, K.I. (1963) *Biochim. Biophys. Acta* **72**, 619-629
17. Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Southern, E.M. (1975) *J. Mol. Biol.* **98**, 503-517
19. Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248-254
20. Yagi, T., Kagamiyama, H., & Nozaki, M. (1981) *Anal. Biochem.* **110**, 146-149
21. Rowsell, E.V., Snell, K., Carnie, J.A., & Al-Tai, A.H. (1969) *Biochem. J.* **115**, 1071-1073
22. Allmansberger, R., Knaub, S., & Klein, A. (1988) *Nucleic Acids Res.* **16**, 7419-7436
23. Brown, J.W., Daniels, C.J., & Reeve, J.N. (1989) *CRC Crit. Rev. Microbiol.* **16**, 287-338
24. Clements, A.P. & Ferry, J.G. (1992) *J. Bacteriol.* **174**, 5244-5250
25. Hamilton, P.T. & Reeve, J.N. (1985) *Mol. Gen. Genet.* **200**, 47-59
26. Possot, O., Sibold, L., & Aubert, J.-P. (1989) *Res. Microbiol.* **140**, 355-371
27. Ovchinnikov, Yu., Egorov, C.A., Aldanova, N.A., Feigina, M. Yu., Lipkin, V.M., Abdulaev, N.G., Grishin, E.V., Kiselev, A.P., Modyanov, N.N., Braunstein, A.E., Polyanovsky, O.L., & Nosikov, V.V. (1973) *FEBS Lett.* **29**, 31-34
28. Doonan, S., Doonan, H.J., Hanford, R., Vernon, C.A., Walker, J.M., Airoldi, L.P., Bossa, F., Barra, D., Carloni, M., Fasella, P., & Riva, F. (1975) *Biochem. J.* **149**, 497-506
29. Kagamiyama, H., Sakakibara, R., Tanase, S., Morino, Y., & Wada, H. (1980) *J. Biol. Chem.* **255**, 6153-6159
30. Oda, T., Miyajima, H., Suzuki, Y., & Ichiyama, A. (1987) *Eur. J. Biochem.* **168**, 537-542
31. Takada, Y., Kaneko, N., Esumi, H., Perdue, P.E., & Danpure, C.J. (1990) *Biochem. J.* **268**, 517-520
32. Duncan, K. & Cogins, J.R. (1986) *Biochem. J.* **234**, 49-57
33. Misrahi, M., Atger, M., & Milgrom, E. (1987) *Biochemistry* **26**, 3975-3982
34. Picot, D., Sandmeier, E., Thaller, C., Vincent, M.G., Christen, P., & Jansonius, J.N. (1991) *Eur. J. Biochem.* **196**, 329-341
35. Saier, M.H. & Jenkins, W.T. (1967) *J. Biol. Chem.* **242**, 101-108
36. Yano, T., Mizuno, T., & Kagamiyama, H. (1993) *Biochemistry* **32**, 1810-1815
37. Ouzounis, C. & Sander, C. (1993) *FEBS Lett.* **322**, 159-164
38. van der Oost, J., van Walraven, H.S., Bogerd, J., Smit, A.B., Ewart, G.D., & Smith, G.D. (1989) *Nucleic Acids Res.* **17**, 10098
39. Ewart, G.D., Reed, K.C., & Smith, G.D. (1990) *Eur. J. Biochem.* **187**, 215-223