Journal of Bacteriology, Mar. 1990, p. 1345–1351
0021-9193/90/031345-07$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 172, No. 3

Purification and Characterization of Thermostable Aspartate Aminotransferase from a Thermophilic *Bacillus* Species

MOON-HEE SUNG,[1†] KATSUYUKI TANIZAWA,[1‡*] HIDEHIKO TANAKA,[1§] SEIKI KURAMITSU,[2] HIROYUKI KAGAMIYAMA,[2] AND KENJI SODA[1]

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611,[1] and Department of Medical Chemistry, Osaka Medical College, Daigaku-cho, Takatsuki, Osaka 569,[2] Japan

Received 17 August 1989/Accepted 29 November 1989

Aspartate aminotransferase (EC 2.6.1.1) was purified to homogeneity from cell extracts of a newly isolated thermophilic bacterium, *Bacillus* sp. strain YM-2. The enzyme consisted of two subunits identical in molecular weight (M_r, 42,000) and showed microheterogeneity, giving two bands with pIs of 4.1 and 4.5 upon isoelectric focusing. The enzyme contained 1 mol of pyridoxal 5′-phosphate per mol of subunit and exhibited maxima at about 360 and 415 nm in absorption and circular dichroism spectra. The intensities of the two bands were dependent on the buffer pH; at neutral or slightly alkaline pH, where the enzyme showed its maximum activity, the absorption peak at 360 nm was prominent. The enzyme was specific for L-aspartate and L-cysteine sulfinate as amino donors and α-ketoglutarate as an amino acceptor; the K_ms were determined to be 3.0 mM for L-aspartate and 2.6 mM for α-ketoglutarate. The enzyme was most active at 70°C and had a higher thermostability than the enzyme from *Escherichia coli*. The N-terminal amino acid sequence (24 residues) did not show any similarity with the sequences of mammalian and *E. coli* enzymes, but several residues were identical with those of the thermoacidophilic archaebacterial enzyme recently reported.

Aspartate aminotransferase (AspAT) (L-aspartate:2-oxoglutarate aminotransferase; EC 2.6.1.1) occurs in virtually all living systems, playing a central role in nitrogen metabolism (4, 5, 24). Eucaryotic cells contain two genetically distinct isoenzymes (cytosolic and mitochondrial AspATs) (4), whereas only a single AspAT has been found in microorganisms (6, 19, 22, 31, 36). Although the degree of amino acid sequence similarity between the heterotopic isoenzymes from various vertebrates is at most 50% (7), X-ray crystallographic studies of the pig cytosolic (1), chicken cytosolic (3, 10), and chicken mitochondrial (12) enzymes revealed that their three-dimensional structures are essentially the same, suggesting that the cytosolic and mitochondrial isoenzymes of AspAT have evolved from a common ancestral enzyme. In marked contrast with detailed studies on the eucaryotic enzymes, the complete amino acid sequences of the procaryotic enzymes have been determined only for the *Escherichia coli* AspAT (15, 16). The overall sequence homology with the eucaryotic enzymes is about 40%. All of the amino acid residues that were proposed from crystallographic studies on the vertebrate enzymes to be essential for the catalysis are also well conserved in the *E. coli* enzyme (15).

Microbial AspATs have been purified from two mesophilic bacteria, *E. coli* (6, 19, 31) and *Pseudomonas putida* (36), and from a yeast, *Saccharomyces cerevisiae* (34), and characterized. The enzymic properties, including molecular weight, number of subunits, absorption spectra, and Michaelis constants for the substrates of the microbial AspATs, are similar to those of the eucaryotic enzymes (35). Recently, the AspAT of a thermoacidophilic archaebacterium, *Sulfolobus solfataricus*, was also purified, and some of its properties were reported (18). However, the partial sequence in its N-terminal region showed no homology with any stretch of known sequence of AspAT from other sources, and the evolutionary relationship between the archaebacterial enzyme and those from eucaryotes and eubacteria remains to be studied.

We have isolated thermophilic eubacteria that belong to the genus *Bacillus* from a sauna dust, and we have demonstrated the occurrence of AspAT in the isolated thermophiles as well as in various other thermophilic bacteria (27). In this paper, we describe purification of AspAT from the thermophilic *Bacillus* sp. strain YM-2 and characterization of its catalytic and structural properties. We provide evidence that shows some sequence similarity in the N-terminal region between the eubacterial and archaebacterial enzymes.

MATERIALS AND METHODS

Culture conditions. *Bacillus* sp. strain YM-2 (27) was grown aerobically at 55°C for 12 h in a medium (160 liters, pH 7.2) containing 0.3% polypeptone, 0.25% yeast extract, 0.1% meat extract, 0.5% glycerol, 0.05% NaCl, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and a trace of biotin (4 ng/ml) with a 200-liter Marubishi Bioengineering MPF-U fermenter. The cells harvested by centrifugation were washed twice with 10 mM potassium phosphate buffer (pH 7.2) and stored at −20°C until used.

Enzyme and protein assays. The enzyme was routinely assayed at 50°C by a modification of the malate dehydrogenase-coupled method (13). The reaction mixture contained 100 μmol of Tris hydrochloride buffer (pH 8.0), 100 μmol of L-aspartate, 10 μmol of α-ketoglutarate, 0.2 μmol of NADH, 0.015 μmol of pyridoxal 5′-phosphate (PLP), 0.1 to 3.0 U of AspAT, and 3 μg (3.6 U) of malate dehydrogenase (Sigma

* Corresponding author.
† Present address: Laboratory of Biochemistry, Genetic Engineering Center, Korea Advanced Institute of Science and Technology, Cheongryang, Seoul, Korea.
‡ Present address: Laboratory of Food Chemistry, Institute of Scientific and Industrial Research, Osaka University, Mihogaoka 8-1, Ibaraki, Osaka 567, Japan.
§ Present address: Laboratory of Applied Gene Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan.

Chemical Co.) in a final volume of 1.0 ml, and the reaction was monitored by the decrease in A_{340} with a Shimadzu MPS-2000 recording spectrophotometer. One unit of AspAT was defined as the amount of enzyme that catalyzed the production of 1 μmol of oxalacetate per min at 50°C. Specific activity was expressed as units per milligram of protein. For determination of kinetic parameters, the reaction rates were measured by a modification of the direct spectrophotometric method (11). The reaction was carried out at 50°C in a mixture (1.0 ml) containing 100 μmol of Tris hydrochloride buffer (pH 8.0), 0.2 to 3.2 μmol of L-aspartate, 0.04 to 2.56 μmol of α-ketoglutarate, 0.015 μmol of PLP, and 1 U of AspAT in a total volume of 1.0 ml. The reaction was monitored by the increase in A_{255}. The molar absorption coefficient of oxalacetate (894/M · cm) was obtained by subtracting the molar A_{255} of α-ketoglutarate. Protein was measured by the method of Lowry et al. (17), with bovine serum albumin as a standard or from the absorption coefficient of the enzyme ($A_{1\,cm}^{1\%}$ at 280 nm, 11.2), estimated by ultracentrifugal analysis with a specific refractive increment for solute protein of 1.837×10^{-3} (21).

Molecular mass determination. Molecular mass was determined at room temperature by high-performance liquid chromatography with a Pharmacia fast-protein liquid chromatography system in two tandem columns of Superose 12 (1 by 30 cm; Pharmacia, Uppsala, Sweden) at a flow rate of 0.3 ml/min and an elution buffer consisting of 50 mM potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl. A calibration curve was made with the following proteins obtained from Oriental Yeast, Osaka, Japan: yeast glutamate dehydrogenase (290,000 daltons [Da]), pig heart lactate dehydrogenase (140,000 Da), yeast enolase (67,000 Da), yeast adenylate kinase (32,000 Da), and horse cytochrome *c* (12,400 Da). The molecular mass of the subunit was estimated by 0.1% sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (14) by using the following standard proteins obtained from Pharmacia: phosphorylase *b* (94,000 Da), bovine serum albumin (67,000 Da), egg albumin (43,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da). Ultracentrifugation was done in a Spinco model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm for the sedimentation velocity experiment and a Rayleigh interference optical system for the sedimentation equilibrium experiment, as described by Van Holde and Baldwin (28). The top speeds of ultracentrifugation for the velocity and equilibrium experiments were 59,780 and 8,225 rpm, respectively. The molecular weight of enzyme was calculated by the method of Nazarian (20).

Isoelectric focusing. Isoelectric focusing was performed on a Phast System (Pharmacia) with Phast Gel IEF media (pH 3 to 9). Electrophoresis was carried out according to the instructions provided by Pharmacia, and a Pharmacia broad pI calibration kit was used for standard proteins.

Amino acid analysis. Amino acid analysis was performed by the method of Spackman et al. (26) with a Beckman 7300 high-performance amino acid analyzer. The enzyme (120 μg) was dialyzed against 5 mM potassium phosphate buffer (pH 7.2) for 48 h and then hydrolyzed at 108°C under reduced pressure for 24, 48, and 72 h in 6 N HCl containing 1% (vol/vol) phenol with a Waters PICO-TAG work station. The hydrolysates were evaporated to dryness and subjected to amino acid analysis in duplicate. Tryptophan was determined spectrophotometrically (8), and cysteine was determined by the Ellman method (23).

N- and C-terminal amino acid sequence analyses. The purified enzyme (0.11 mg; 2.62 nmol of subunit in 5 mM potassium phosphate buffer [pH 7.2]) was used directly for the N-terminal sequence analysis by automated Edman degradation with an Applied Biosystems 470A gas-liquid-phase protein sequencer. The phenylthiohydantoin amino acid derivatives were separated and identified by an on-line phenylthiohydantoin analyzer model 120A (Applied Biosystems) with a PTH-C_{18} column. The initial and averaged repetitive yields (up to the 24th cycle) were about 45 and 96%, respectively. The C-terminal amino acid residues were analyzed after carboxypeptidase P digestion (37). The enzyme (0.154 mg; 3.67 nmol of subunit) was lyophilized, dissolved in 0.2 ml of 0.1 M sodium acetate buffer (pH 3.7) containing 0.02% Triton X-100, and digested at 30°C with 43 pmol of carboxypeptidase P (Takara Shuzo, Kyoto, Japan). At various intervals, 35-μl samples were withdrawn, mixed with 10 μl of 1 N NaOH, dried by evaporation, and dissolved in 0.1 ml of 0.2 M sodium citrate buffer (pH 2.2). The amino acids released by the digestion were determined by automatic amino acid analysis.

Purification of enzyme. All operations were carried out at 0 to 4°C, and potassium phosphate buffer (pH 7.2) containing 20 μM PLP and 0.01% 2-mercaptoethanol was used as the buffer throughout the purification unless otherwise specified.

(i) Step 1. Washed cells (about 1.0 kg, wet weight) were suspended in 2.5 liters of 20 mM buffer supplemented with 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone, and disrupted at 4°C with a 20-KHz Seiko-Biom'c model 7500 ultrasonic oscillator. The intact cells and cell debris were removed by centrifugation.

(ii) Step 2. To the cell extract (3,410 ml) was added 5.7 g of protamine sulfate with stirring. After 20 min, the precipitate formed was removed by centrifugation. The supernatant (3,350 ml) was concentrated to about 500 ml with a Millipore XX42 ultrafiltration system and dialyzed against 2,500 volumes of 10 mM buffer containing 0.2 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, and 5 μM *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone.

(iii) Step 3. The enzyme solution was applied to a DEAE-Toyopearl 650M column (8 by 39 cm; Tosoh, Tokyo, Japan) equilibrated with 10 mM buffer. After the column was washed thoroughly with the buffer, the enzyme was eluted with the buffer containing 0.24 M KCl. The active fractions were pooled and concentrated to about 90 ml by ultrafiltration.

(iv) Step 4. The enzyme was applied in 6-ml portions to a TSK DEAE-5PW column (2.15 by 15 cm; Tosoh) on a Tosoh HLC-837 preparative liquid chromatograph. The enzyme was eluted with 10 mM buffer containing 0.21 M KCl. The active fractions from each chromatography were combined and concentrated by ultrafiltration with an Amicon PM-10 membrane.

(v) Step 5. The enzyme solution (40 ml) was brought to 35% saturation with ammonium sulfate and then applied to a Butyl-Toyopearl column (3.6 by 24.5 cm; Tosoh) equilibrated with 10 mM buffer containing 35% saturated ammonium sulfate. After a wash with 1 liter of the same buffer, the enzyme was eluted with a 1-liter linear gradient of saturated ammonium sulfate (35 to 15%) in 10 mM buffer. The active fractions (180 ml) were pooled and concentrated by ultrafiltration.

(vi) Step 6. The enzyme solution (14 ml) was brought to 35% saturation with ammonium sulfate and put on a Phenyl-superose HR 5/5 column (0.5 by 5 cm; Pharmacia) in a fast-protein liquid chromatography system. The elution was

TABLE 1. Summary of purification of aspartate aminotransferase from *Bacillus* sp. strain YM-2

Step and prepn	Total protein (mg)	Sp act (U/mg)	Total activity (U)	Yield (%)
1. Crude extract	56,800	0.15	8,710	100
2. Protamine sulfate	46,500	0.30	14,000	161
3. DEAE-Toyopearl	7,340	1.78	13,100	150
4. DEAE-5PW	2,870	3.16	9,070	104
5. Butyl-Toyopearl	69.4	120	8,300	95
6. Phenylsuperose	18.7	218	4,080	47
7. Hydroxyapatite	12.0	220	2,640	30

carried out at a flow rate of 0.8 ml/min with a 20-min linear gradient of saturated ammonium sulfate (25 to 17.5%) in 10 mM buffer. The active fractions (80 ml) were pooled and concentrated by ultrafiltration.

(vii) Step 7. The enzyme solution (20 ml) was dialyzed against 5 mM buffer and applied to a hydroxyapatite column (0.75 by 10 cm; Toa Nenryo, Tokyo, Japan) equipped on an LKB Ultrochrome GTi high-pressure liquid chromatography system (Bromma, Sweden). The enzyme was eluted at a flow rate of 0.8 ml/min with a 45-min linear gradient of potassium phosphate buffer (pH 7.2) (5 to 100 mM) containing 0.3 mM $CaCl_2$. The enzyme thus purified was concentrated to >10 mg/ml by ultrafiltration, dialyzed against 100 volumes of 10 mM buffer, and stored at −20°C.

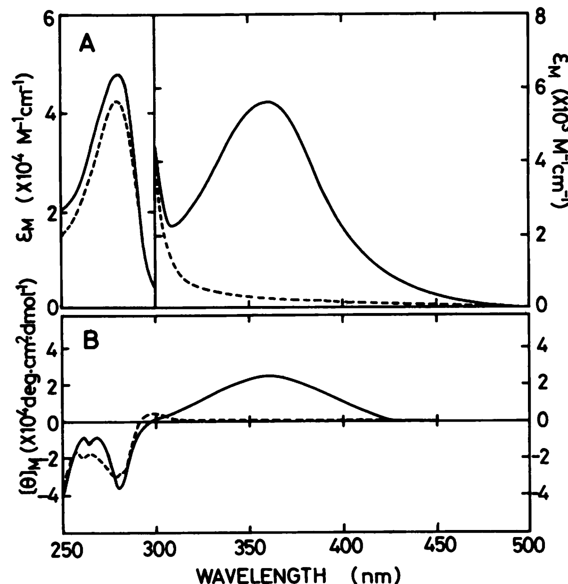

FIG. 1. Absorption (A) and circular dichroic (B) spectra of AspAT from *Bacillus* sp. strain YM-2. Spectra were taken in 50 mM potassium phosphate buffer (pH 7.2) at protein concentrations of 1.0 to 2.5 mg/ml and corrected for molar absorption coefficients (ε_M) and ellipticities ($[\theta]_M$) based on a subunit molecular weight of 42,000. Symbols: (——) holoenzyme, (– – –) apoenzyme.

RESULTS

Purification of AspAT from *Bacillus* sp. strain YM-2. Purification of the enzyme resulted in an approximately 1,400-fold enhancement of specific activity. Typical results of the purification procedure are shown in Table 1. The significant increase in total activity by the protamine sulfate treatment may be due to the removal of an inhibitory substance(s) present in the cell extract. The purified enzyme showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) and had a specific activity of 220 μmol/min per mg, which is comparable to that of the *E. coli* enzyme (200 μmol/min per mg at 37°C) (31).

Molecular mass and subunit structure. The enzyme sedimented as a symmetrical peak during the sedimentation velocity experiment, and its sedimentation coefficient corrected to water at 20°C was calculated to be 4.94S. The molecular mass of the native enzyme was determined to be 98,000 ± 2,000 Da by gel filtration on two tandemly connected Superose 12 columns. A molecular mass of 97,000 ± 4,000 Da was obtained by sedimentation equilibrium with a partial specific volume of 0.743, which was calculated from the amino acid composition. The subunit structure was examined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (14). The denatured enzyme migrated as a single band of stained protein, and its molecular mass was estimated to be about 42,000 Da on the basis of its mobility relative to those of standard proteins. Thus, the enzyme appears to be a dimer composed of two identical subunits, the structure of which is common to all AspATs reported so far. However, the noticeable discrepancy between the molecular mass determined with the native enzyme and that predicted from the subunit (42,000 Da × 2) is unexplained.

Isoelectric point. The purified enzyme showed microheterogeneity when analyzed by isoelectric focusing on polyacrylamide gels in the pH range from 3.0 to 9.0; two bands corresponding to pIs of 4.5 (major band) and 4.1 (minor band) were observed. The activity staining with L-cysteine sulfinate as a substrate (33) indicated that both bands were catalytically active. Thus, both of the enzymes with pIs of 4.1 and 4.5 are probably the subforms of AspAT. These subforms might be produced by deamidation of asparaginyl or glutaminyl residues in the protein, as has been reported for the subforms of the mammalian cytosolic enzyme (30). In accordance with this suggestion, we found that two nonadecapeptides obtained by trypsin digestion of the enzyme (a mixture of the subforms), which were slightly separated from each other by reversed-phase liquid chromatography, showed an identical amino acid sequence, except that one peptide has an aspartyl residue at the position where the other has an asparaginyl residue (data not shown).

Spectrophotometric properties of the enzyme-bound PLP. The enzyme was found to contain 1 mol of PLP per mol of subunit by the fluorophotometric method with KCN (2). The enzyme exhibited an absorption maximum at 360 nm due to the bound cofactor (Fig. 1A), and the spectrum changed significantly by the buffer in the pH range of 4.9 to 8.5 (Fig. 2). The peak at 360 nm was prominent at neutral and slightly alkaline pH, where the enzyme showed its maximum activity (see below), whereas at acidic pH the absorption maximum shifted to 415 nm. This pH-dependent spectral change is common to all AspATs reported so far, with the exception of the enzyme from the archaebacterium *S. solfataricus* (18). The molar absorption coefficients were calculated to be 47,000/M · cm at 280 nm, 5,560/M · cm at 360 nm (pH 7.4), and 4,800/M · cm at 415 nm (pH 4.9) on the basis of the subunit molecular weight (42,000). Since the spectral curves at various pH gave an isosbestic point at 384 nm (Fig. 2), the bound cofactor is in equilibrium of two chemical species absorbing at different wavelengths. It has been suggested that these two species are derived from the difference in ionization state of the nitrogen atom of the internal Schiff

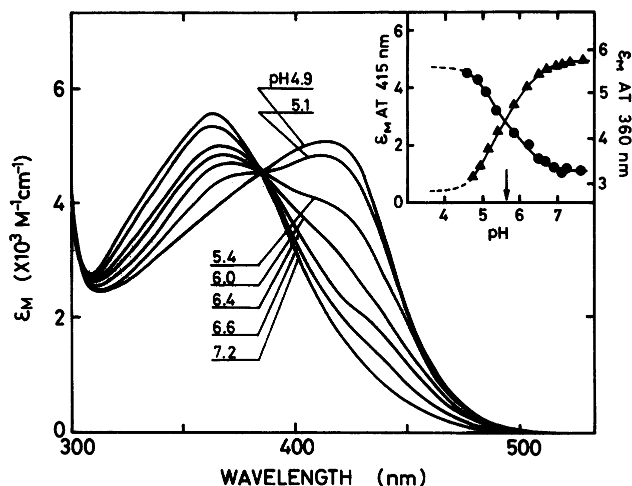

FIG. 2. Effect of pH on absorption spectra of the enzyme-bound PLP. The buffers used were 0.1 M sodium acetate (pH 4 to 6), potassium phosphate (pH 6 to 8), and Tris hydrochloride (pH 7 to 8.5). Inset: Determination of the pK value of the Schiff base protonation. Molar absorption values (ε_Ms) at 360 nm (▲) and 415 nm (●) were plotted against pH.

base formed between the aldehyde group of PLP and an amino group of a lysyl residue of the enzyme (4). By plotting the molar absorption values against pH (Fig. 2, inset), the pK of the protonation and deprotonation was determined to be about pH 5.7.

The bound PLP exhibited a positive peak at 360 nm in the circular dichroic spectrum (Fig. 1B), which also varied with pH (data not shown), corresponding with the absorption spectrum. The molar ellipticity $[\theta]_M$ was calculated to be about 24,000 deg · cm^2 per dmol of the bound PLP (subunit). The apoenzyme, which was obtained by incubation of the enzyme with 50 mM L-cysteine sulfinate followed by precipitation with an acidic ammonium sulfate solution (25), showed no peak at 300 to 500 nm in absorption and circular dichroic spectra (Fig. 1). By spectrophotometric and catalytic titrations of the apoenzyme with PLP, the apparent K_d for PLP was determined to be about 63 nM.

Amino acid composition. The amino acid composition of the purified enzyme is given in Table 2. The predominant residues of the enzyme protein are glutamic acid, alanine, aspartic acid, and isoleucine. No other striking features for an enzyme from thermophiles were observed, except that no cysteinyl residue and only a few histidyl and tryptophanyl residues are contained. The total of integral numbers of each amino acid residue gave a calculated molecular mass of about 45,000 Da, which corresponds with the value determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Terminal sequence analyses. The N-terminal sequence (24 amino acid residues) of the enzyme protein was determined by automated Edman degradation: Met-Lys-Glu-Leu-Leu-Ala-Asn-Arg-Val-Lys-Thr-Leu-Thr-Pro-Ser-Thr-Thr-Leu-Ala-Ile-Thr-Ala-Lys-Ala-. Methionine was found as the N-terminus in a relatively high initial yield (about 45%, based on the amount of subunit). Only one major phenylthiohydantoin derivative of amino acids was detected in each cycle of the Edman degradation. This indicates that the enzyme is composed of two identical polypeptide chains.

The C-terminal amino acid residue was analyzed by measurement of amino acids released after the carboxypeptidase P digestion. The first residue released by the digestion was

TABLE 2. Amino acid composition of aspartate aminotransferase from *Bacillus* sp. strain YM-2

Amino acid	Mol%	Mol/mol[a] of subunit
Cysteine[b]	0.00	0
Aspartic acid	8.87	36
Threonine[c]	6.65	27
Serine[c]	6.65	27
Glutamic acid	13.55	55
Proline	5.67	23
Glycine	6.40	26
Alanine	10.84	44
Valine	6.16	25
Methionine	1.72	7
Isoleucine	8.62	35
Leucine	6.90	28
Tyrosine	4.43	18
Phenylalanine	2.71	11
Lysine	6.65	27
Histidine	0.74	3
Arginine	2.71	11
Tryptophan[d]	0.74	3

[a] Values were calculated on the basis of a subunit molecular weight of 42,000.

[b] The amount of cysteine was determined by the Ellman method as described previously (23).

[c] The values for serine and threonine were obtained by extrapolation to zero hydrolysis time.

[d] The tryptophan content was estimated spectrophotometrically by the method of Edelhoch (8). The tyrosine content estimated by this method agreed well with the amino acid analysis value.

lysine, which was calculated to be 0.8 to 1.1 mol/mol of subunit, followed by the release of valine, phenylalanine, arginine, and some others (Fig. 3). This suggests that the enzyme has a single C terminus, the putative sequence of which is -(Ile)-(Leu)-(Arg)-(Phe)-Val-Lys-COOH.

Effects of pH and temperature on stability and activity. The purified enzyme was found to be stable; it lost no activity when incubated at 37°C for 1 h at pH 6.0 to 11.0 in various buffers. When heated for 20 min in 10 mM potassium phosphate buffer (pH 7.2), the enzyme was stable at up to 70°C. After incubation at 80°C for 20 min, the remaining enzyme activity was about 65% of the initial activity.

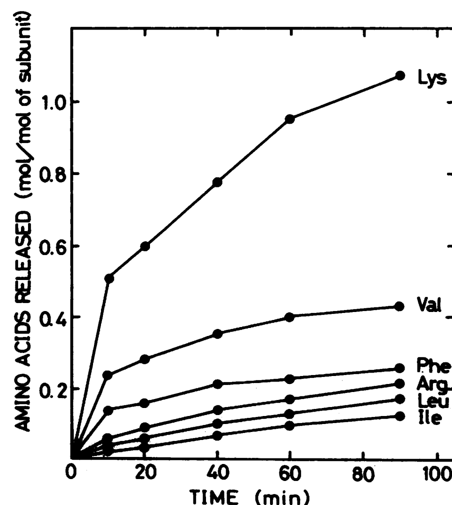

FIG. 3. Analysis of C-terminal amino acid residues of AspAT from *Bacillus* sp. strain YM-2 by carboxypeptidase P digestion. The amino acids released by the digestion at various incubation times were measured with an amino acid analyzer.

TABLE 3. Substrate specificities of aspartate aminotransferase from *Bacillus* sp. strain YM-2

Amino acid[a]	Relative activity
L-Aspartic acid	100
L-Cysteinesulfinic acid	199
L-α-Aminoadipic acid	1.7
L-Kynurenine	0.9
L-Cysteine	0.9
L-Alanine	0.3
L-Methionine	0.2
L-Arginine	0.2
L-Leucine	0.1
L-Tryptophan	0.1

[a] Amino acids were used at a concentration of 50 mM except for L-kynurenine (20 mM) and L-tryptophan (20 mM). The reaction was carried out at 50°C for 10 min in the reaction system containing 20 mM α-ketoglutarate and an amino acid to be tested, and the amount of glutamic acid produced was determined with an automatic amino acid analyzer. The following amino acids were inert (relative activity of less than 0.1): glycine, L-phenylalanine, L-tyrosine, L-isoleucine, L-proline, L-serine, L-homoserine, L-threonine, L-asparagine, L-histidine, L-glutamine, L-valine, L-norvaline, L-lysine, and D-aspartic acid.

The enzyme exhibited maximal activity at pH 7.2 when examined in the presence of 0.1 M sodium acetate, potassium phosphate, Tris hydrochloride, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and *N*,*N*-bis(2-hydroxyethyl)-glycine buffers. When the enzyme was assayed at various temperatures, maximal activity was found at 70°C. The reaction rate increased as the temperature was raised from 20 to 70°C and declined over 75°C.

Substrate specificity. The ability of the enzyme to catalyze the transamination between various amino acids and α-ketoglutarate was examined in the presence of kinetically saturating amounts (20 to 50 mM) of the substrates (Table 3). L-Cysteine sulfinate was a better amino donor substrate than L-aspartate, probably because the aminotransferase reaction of L-cysteine sulfinate is irreversible (32). Aromatic amino acids such as L-tryptophan and L-phenylalanine were negligibly active as amino donors. The amino acceptor specificity also was studied with L-aspartate as an amino donor and a variety of α-keto acids. Besides α-ketoglutarate (relative activity, 100), α-ketoadipate (0.5), α-keto-*n*-butyrate (0.5), and α-keto-*n*-valerate (0.4) served slightly as substrates. Pyruvate, α-keto-*iso*-valerate, α-keto-*iso*-caproate, *p*-hydroxy-β-phenylpyruvate, glyoxylate, α-keto-*n*-caproate, β-indolepyruvate, and homophenylpyruvate were not substrates.

Kinetic mechanism. A series of steady-state kinetic analyses was carried out to investigate the reaction mechanism. Initial-velocity studies were performed at 50°C by the method of Velick and Vavra (29). Double-reciprocal plots of initial velocity against concentrations of L-aspartate and α-ketoglutarate in the presence of various fixed concentrations of α-ketoglutarate and L-aspartate, respectively, gave two sets of parallel lines (Fig. 4). The reaction proceeds via the "ping-pong bi-bi" mechanism (29), as has been demonstrated for all other aminotransferases. The Michaelis constants for L-aspartate and α-ketoglutarate were calculated to be 3.0 and 2.6 mM, respectively, from the secondary plots of intercept versus reciprocal concentrations of the other substrate. The K_ms were higher than those of AspATs from other sources.

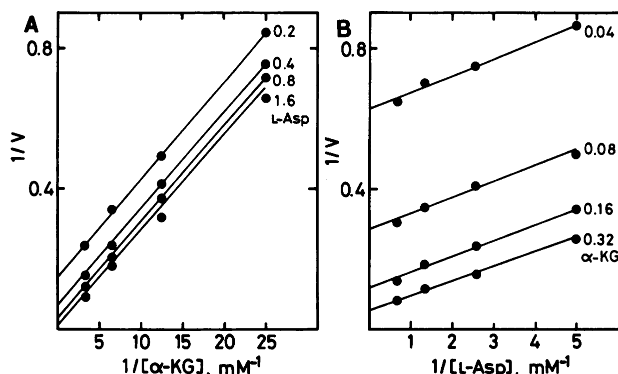

FIG. 4. Effects of concentrations of L-aspartate and α-ketoglutarate on the initial velocities. The reaction mixture contained variable amounts of the substrates as indicated, and the enzyme was assayed as described in the text. (A) Double-reciprocal plots of velocity versus α-ketoglutarate (α-KG) concentration at several fixed concentrations (millimolar) of L-aspartate (L-Asp). (B) Double-reciprocal plots of velocity versus L-aspartate concentration at several fixed concentrations (millimolar) of α-ketoglutarate.

DISCUSSION

We have described in detail the physicochemical and catalytic properties of AspAT that was purified to homogeneity from cell extracts of a newly isolated thermophile, *Bacillus* sp. strain YM-2 (27). The purified enzyme was proven to have higher thermostability by about 20°C than AspAT from *E. coli* (31). However, basic enzymological properties, including molecular mass, number of subunit, absorption and circular dichroism spectra, and catalytic activity, of the *Bacillus* sp. strain YM-2 enzyme are similar to those of the other microbial and eucaryotic enzymes. Noteworthy slight differences are that the *Bacillus* sp. strain YM-2 enzyme has much lesser activity for aromatic amino acids than the *E. coli* and mammalian enzymes, and that Michaelis constants for substrates are higher by severalfold than those of the enzymes from other sources. The amino acid composition of the *Bacillus* AspAT is also different; a statistical analysis of the amino acid compositions on a mole percent basis by the method of Harris et al. (9) yielded rather high deviation functions (*D* values) between the thermostable AspAT and the enzymes from other sources (0.095 with the *E. coli* enzyme, 0.086 with the chicken mitochondrial enzyme, 0.083 with the pig mitochondrial enzyme, 0.093 with the chicken cytosolic enzyme, and 0.091 with the pig cytosolic enzyme). The *D* values higher than 0.05 are thought to be insignificant for showing a structural similarity between any two enzymes (9).

Although the C-terminal amino acid residue of the *Bacillus* sp. strain YM-2 AspAT (Lys) was the same as that of vertebrate mitochondrial enzymes, the N-terminal amino acid sequence was markedly different from others (Fig. 5). Crystallographic studies of the mammalian enzymes revealed that the N-terminal region forms an indistinct secondary structure, protruding into the solvent in the absence of substrate, whereas the C-terminal region is situated in the rigid structure of the enzyme, constituting a helical segment (1, 3, 10, 12). This is consistent with the idea that the N-terminal region of AspATs generally has lower sequence similarity than the other part of the entire sequence, and therefore the residues in the N-terminal region are less conservative during the course of evolution. We recently cloned and sequenced the *Bacillus* gene coding for AspAT; we found that the entire amino acid sequence deduced from DNA was also quite dissimilar to those of *E. coli* and mammalian enzymes (Sung et al., unpublished results). The

cAspAT pig	APPSVFAEVPQAQPVLVFKLIADFREDPDP-
cAspAT chicken	AASIFAAVPRAPPVAVFKLTADFREDGDS-
mAspAT pig	SSWWAHVEMGPPDPILGVTEAFKRDTNS-
mAspAT chicken	SSWWSHVEMGPPDPILGVTEAFKRDTNS-
AspAT *E. coli*	MFENITAAPADPILGLADLFRADERP-
AspAT *S. solfataricus*	VSLLDFNGNMSQVTGETTLLYK-
AspAT *Bacillus* sp. YM-2	MKELL-ANRVKT-LTPSTTLAITAKA-

FIG. 5. Comparison of the N-terminal amino acid sequence of AspAT from *Bacillus* sp. strain YM-2 with those of the enzymes from other sources. N-terminal partial sequences of chicken and pig cytosolic (cAspAT) and mitochondrial (mAspAT) enzymes and of the *S. solfataricus* enzyme were cited from references 7 and 18. Identical residues among more than two sequences are shown in boldface type. Gaps (hyphens) were inserted for maximum matching.

overall sequence identity (<8%) between the *Bacillus* AspAT and *E. coli* or mammalian enzymes was much lower than that (about 40%) between *E. coli* and mammalian enzymes. These findings suggest that if divergent evolution is the case for the *Bacillus* AspAT, the genes encoding the *Bacillus* and *E. coli* enzymes might have diverged from each other in the very early evolutionary stage before the emergence of the eucaryotic AspATs (7).

It is noteworthy that several residues in the N-terminal region of the *Bacillus* enzyme are identical with those of the thermoacidophilic archaebacterial enzyme, which is extraordinary thermostable (18) (Fig. 5). This might suggest either that convergent evolution is operative in these thermostable AspATs or that these enzyme genes diverged at around the same time as the emergence of archaebacteria and eubacteria. Thus, it is of particular interest to compare AspATs from various sources on the basis of the complete primary structure. Details of gene cloning and DNA sequencing for the *Bacillus* thermostable AspAT will be reported shortly.

LITERATURE CITED

1. **Arnone, A., P. H. Rogers, C. C. Hyde, P. D. Briley, C. M. Metzler, and D. E. Metzler.** 1985. Pig cytosolic aspartate aminotransferase: the structures of the internal aldimine, external aldimine, and ketimine and of the β subform, p. 138–155. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
2. **Bonavita, V.** 1960. The reaction of pyridoxal 5-phosphate with cyanide and its analytical use. Arch. Biochem. Biophys. **88:** 366–372.
3. **Borisov, V. V., S. N. Borisova, G. S. Kachalova, N. I. Sosfenow, and B. K. Vainshtein.** 1985. X-ray studies of chicken cytosolic aspartate aminotransferase, p. 155–164. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
4. **Braunstein, A. E.** 1973. Amino group transfer, p. 379–481. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. 9, part B. Academic Press, Inc., New York.
5. **Braunstein, A. E.** 1985. Transamination and transaminases, p. 2–19. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
6. **Chesne, S., and J. Pelmont.** 1973. Glutamate-oxaloacétate transaminase d'*Escherichia coli*: I-purification et spécificité. Biochimie **55:**237–244.
7. **Christen, P., U. Graf-Hausner, F. Bossa, and S. Doonan.** 1985. Comparison of covalent structures of the isoenzymes of aspartate aminotransferase, p. 173–185. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
8. **Edelhoch, H.** 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry **6:**1948–1954.
9. **Harris, C. E., R. D. Kobes, D. C. Teller, and W. J. Rutter.** 1969. The molecular characteristics of yeast aldolase. Biochemistry **8:**2442–2454.
10. **Harutyunyan, E. G., V. N. Malashkevich, V. M. Kochkina, and Y. M. Torchinsky.** 1985. Three-dimensional structure of the complex of chicken cytosolic aspartate aminotransferase with 2-oxoglutarate, p. 164–173. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
11. **Henson, C. P., and W. W. Cleland.** 1964. Kinetic studies of glutamic oxaloacetic transaminase isozymes. Biochemistry **3:** 338–345.
12. **Jansonius, J. N., G. Eichele, G. C. Ford, D. Picot, C. Thaller, and M. G. Vincent.** 1985. Spatial structure of mitochondrial aspartate aminotransferase, p. 110–138. *In* P. Christen and D. E. Metzler (ed.), Transaminases. John Wiley & Sons, Inc., New York.
13. **Karmen, A.** 1955. A note on the spectrophotometric assay of glutamic oxalacetic transaminase in human blood serum. J. Clin. Invest. **34:**131–133.
14. **King, J., and U. K. Laemmli.** 1971. Polypeptides of the tail fibers of bacteriophage T4. J. Mol. Biol. **62:**465–477.
15. **Kondo, K., S. Wakabayashi, and H. Kagamiyama.** 1987. Structural studies on aspartate aminotransferase from *Escherichia coli*. Covalent structure. J. Biol. Chem. **262:**8648–8659.
16. **Kondo, K., S. Wakabayashi, T. Yagi, and H. Kagamiyama.** 1984. The complete amino acid sequence of aspartate aminotransferase from *Escherichia coli*: sequence comparison with pig isoenzymes. Biochem. Biophys. Res. Commun. **122:**62–67.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
18. **Marino, G., G. Nitti, M. I. Arnone, G. Sannia, A. Gambacorta, and M. De Rosa.** 1988. Purification and characterization of aspartate aminotransferase from the thermoacidophilic archaebacterium *Sulfolobus solfataricus*. J. Biol. Chem. **263:**12305–12309.
19. **Mavrides, C., and W. Orr.** 1975. Multispecific aspartate and aromatic amino acid aminotransferases in *Escherichia coli*. J. Biol. Chem. **250:**4128–4133.
20. **Nazarian, G. M.** 1968. Determination of molecular weight from the interference pattern of sedimentation equilibrium in the ultracentrifuge. Anal. Chem. **40:**1766–1769.
21. **Perlmann, G. E., and L. G. Longsworth.** 1948. The specific refractive increment of some purified proteins. J. Am. Chem. Soc. **70:**2719–2724.
22. **Powell, J. T., and J. F. Morrison.** 1978. The purification and properties of the aspartate aminotransferase and aromatic-amino-acid aminotransferase from *Escherichia coli*. Eur. J. Biochem. **87:**391–400.
23. **Riddles, P. W., R. L. Blakeley, and B. Zerner.** 1979. Ellman's reagent: 5,5′-dithiobis(2-nitrobenzoic acid)—a reexamination. Anal. Biochem. **94:**75–81.
24. **Safer, B., and J. R. Williamson.** 1973. Mitochondrial-cytosolic interactions in perfused rat heart. Role of coupled transamination in repletion of citric acid cycle intermediates. J. Biol. Chem. **248:**2570–2579.
25. **Scardi, V., P. Scotto, M. Iaccarino, and E. Scarano.** 1963. The binding of pyridoxal 5-phosphate to aspartate aminotransferase of pig heart. Biochem. J. **88:**172–175.
26. **Spackman, D. H., W. H. Stein, and S. Moore.** 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30:**1190–1206.
27. **Sung, M.-H., K. Tanizawa, Y. Masu, H. Tanaka, and K. Soda.** 1987. Bacterial aspartate aminotransferase: its occurrence in thermophilic bacteria, and the isolation and identification of new thermophiles. Agric. Biol. Chem. **52:**269–270.
28. **Van Holde, K. E., and R. L. Baldwin.** 1958. Rapid attainment of sedimentation equilibrium. J. Phys. Chem. **62:**734–743.
29. **Velick, S. F., and J. Vavra.** 1962. A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. J. Biol. Chem. **237:**2109–2122.
30. **Williams, J. A., and R. A. John.** 1979. Generation of aspartate aminotransferase multiple forms by deamidation. Biochem. J. **177:**121–127.
31. **Yagi, T., H. Kagamiyama, K. Motosugi, M. Nozaki, and K.**

Soda. 1979. Crystallization and properties of aspartate aminotransferase from *Escherichia coli* B. FEBS Lett. **100**:81–84.

32. **Yagi, T., H. Kagamiyama, and M. Nozaki.** 1979. Cysteine sulfinate transamination activity of aspartate aminotransferases. Biochem. Biophys. Res. Commun. **90**:447–452.
33. **Yagi, T., H. Kagamiyama, and M. Nozaki.** 1981. A sensitive method for the detection of aspartate:2-oxoglutarate aminotransferase activity on polyacrylamide gels. Anal. Biochem. **110**:146–149.
34. **Yagi, T., H. Kagamiyama, and M. Nozaki.** 1982. Aspartate: 2-oxoglutarate aminotransferase from baker's yeast: crystallization and characterization. J. Biochem. **92**:35–43.
35. **Yagi, T., H. Kagamiyama, M. Nozaki, and K. Soda.** 1985. Glutamate-aspartate transaminase from microorganisms. Methods Enzymol. **113**:83–89.
36. **Yagi, T., M. Toyosato, and K. Soda.** 1976. Crystalline aspartate aminotransferase from *Pseudomonas striata*. FEBS Lett. **61**: 34–37.
37. **Yokoyama, S., A. Oobayashi, O. Tanabe, S. Sugawara, E. Araki, and E. Ichishima.** 1974. Production and some properties of a new type of acid carboxypeptidase of *Penicillium* molds. Appl. Microbiol. **27**:953–960.