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Cloning and Expression of the Pyridoxal 5′-Phosphate–Dependent Aspartate Racemase Gene from the Bivalve Mollusk *Scapharca broughtonii* and Characterization of the Recombinant Enzyme

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D-Aspartate is present at high concentrations in the tissues of *Scapharca broughtonii*, and its production depends on aspartate racemase. This enzyme is the first aspartate racemase purified from animal tissues and unique in its pyridoxal 5′-phosphate (PLP)–dependence in contrast to microbial aspartate racemases thus far characterized. The enzyme activity is markedly increased in the presence of AMP and decreased in the presence of ATP. To analyze the structure–function relationship of the enzyme further, we cloned the cDNA of aspartate racemase, and then purified and characterized the recombinant enzyme expressed in *Escherichia coli*. The cDNA included an open reading frame of 1,017 bp encoding a protein of 338 amino acids, and the deduced amino acid sequence contained a PLP-binding motif. The sequence exhibits the highest identity (43–44%) to mammalian serine racemase, followed mainly by threonine dehydratase. These relationships are fully supported by phylogenetic analyses of the enzymes. The active recombinant aspartate racemase found in the *Escherichia coli* extract represented about 10% of total bacterial protein and was purified to display essentially identical physicochemical and catalytic properties with those of the native enzyme. In addition, the enzyme showed a dehydratase activity toward L-*threo*-3-hydroxyaspartate, similar to the mammalian serine racemase that produces pyruvate from D- and L-serine.

Key words: aspartate racemase, D-aspartate, pyridoxal 5′-phosphate, *Scapharca broughtonii*.

Abbreviations: PLP, pyridoxal 5′-phosphate; RACE, rapid amplification of cDNA ends; AR, alanine racemase; AspR, aspartate racemase; GR, glutamate racemase; SR, serine racemase; SDH, serine dehydratase; TDH, threonine dehydratase; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

D-Amino acids have been discovered in a wide variety of living organisms including mammals and humans, where L-amino acids are generally predominant. D-Aspartate is one of the most abundantly found D-amino acids and attracts attention because of its significant presence in mammalian tissues related to neural and endocrine functions (*1*, *2*), as well as D-serine, which occurs at high levels in mammalian brain and is an endogenous ligand of *N*-methyl-D-aspartate receptors (*3*–*5*). The biosynthesis of D-serine has been shown to depend on pyridoxal 5′-phosphate (PLP)–dependent serine racemase, which was first purified from mouse brain and cloned from the brains of various mammals (*6*–*9*). On the other hand, mechanisms for the biosynthesis of D-aspartate in mammalian tissues have not been elucidated yet, although many findings support its actual production (*10*), and this production is suggested to depend possibly on aspartate racemase based on the observation that D-aspartate is produced from L-aspartate in rat cerebral cultures and that the production is inhibited by an inhibitor of PLP-dependent enzymes (*11*). Apart from mammalian tissues, it has been shown that D-aspartate in the bivalve mollusk *Scapharca broughtonii* is indeed produced by the action of aspartate racemase, which is the only example of an animal aspartate racemase so far purified (*12*). Moreover, this enzyme is PLP-dependent, distinct from aspartate racemases purified and/or cloned from various microorganisms (*13*–*15*), and, in this respect, rather similar to mammalian serine racemase. In addition, the bivalve enzyme as well as the serine racemase are sensitive to adenine nucleotides as shown by the finding that the latter is activated by ATP (*16*, *17*), while the activity of the former is increased and decreased by AMP and ATP, respectively (*18*), suggesting that these enzymes possess binding sites for these nucleotides. Further characterization and elucidation of the structure–function relationship of the aspartate racemase, and comparison of the enzyme with mammalian serine racemase in detail may provide useful clues for understanding the mechanisms of D-aspartate production in mammalian tissues. To obtain suitable amounts of the enzyme for such studies, we chose to clone the enzyme, since it is very difficult to purify substantial amounts of the enzyme from bivalve tissues (*12*).

In the present paper, we report the cloning and characterization of the full-length cDNA encoding foot muscle aspartate racemase of *S. broughtonii*, together with the

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expression of the recombinant enzyme in *Escherichia coli* and its purification and characterization.

MATERIALS AND METHODS

Materials—Live specimens of blood ark shell, *S. broughtonii*, cultured in Miyagi Prefecture, Japan, were purchased from a fish market and kept in seawater bubbled with air. The shells were opened and the more intensely colored outside part (~46% of the total mass) was removed and frozen in liquid nitrogen and stored at –80°C until use. D-Aspartate was a generous gift from Tanabe Pharmaceuticals. L-*threo*-3-Hydoxyaspartic acid was purchased from Tocris Cookson, Ltd. (Bristol, UK). Restriction endonucleases and DNA-modifying enzymes were from Takara Bio (Kyoto, Japan) or Toyobo (Osaka, Japan). The Blue Sepharose 6 FF column, Mono S HR column and HyPrep Sephacryl S-100 HR column were from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and purchased from commercial sources.

Bacterial Strains, Plasmids, and Cultivation—E. coli DH5α was used to clone the enzyme-coding gene, and *E. coli* BL21 (DE3) (Novagen, Madison, WI) was used for overexpression of the enzyme. *E. coli* cells were cultured in LB medium [1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride (w/v)] or 2× YT [1.6% tryptone , 1% yeast extract, and 0.5% sodium chloride (w/v)] containing a suitable antibiotic. Plasmids, pT7Blue T-vector (Novagen), pCR4Blunt TOPO (Invitrogen, Carlsbad, CA) and pET25b (Novagen) were used as the cloning and expression vectors.

Purification of Aspartate Racemase from the Foot Muscle of S. broughtonii and Internal Amino Acid Sequence Analysis—Aspartate racemase was purified from the *S. broughtonii* foot muscle stored frozen by a procedure that included sodium sulfate fractionation and Blue Sepharose column chromatography as previously described (*12*). The purified enzyme (225 μg) was digested at 25°C for 12 h with 80 μg of lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan) in a reaction mixture comprising 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine hydrochloride, and the resulting peptides were resolved by reverse phase high performance liquid chromatography (HPLC) on a capcell Pac C-8 column (0.2 mm × 15 cm) (Shiseido, Tokyo, Japan) using a 2–80% acetonitrile gradient in 0.06% trifluoroacetic acid. Five peptides were obtained and applied to a Polybrene-coated trifluoroacetic acid–activated precycled glass fiber filter for amino acid sequencing and analyzed with a Shimadzu PPSQ-21 protein sequencer.

Isolation of cDNA Encoding Aspartate Racemase—Total RNA was extracted from the *S. broughtonii* foot muscle stored frozen using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Poly $(A)^+$ RNA was purified from total RNA using an Oligotex™-dT30 <Super> mRNA Purification kit (Takara) according to the manufacturer's instructions. Single-strand cDNA synthesis was performed with a ThermoScript RT-PCR System (Invitrogen) from 5 μg of total RNA using an oligo dT primer and ThermoScript reverse transcriptase (Invitrogen). A forward degenerate primer pS3 [5′-ATGGAAAA(C/T)TACGG(A/G/C/T)GC(A/C/T)GA-3′] and a reverse degenerate primer pA1 [5′-CCTTC(G/T)GGTTC(A/G/T)ACIA(A/G)(A/G)AA-3′] were designed based on the determined internal amino acid sequences MENYGAE and FLVEPEG (Fig. 1), respectively, and were used for polymerase chain reactions (PCR) to obtain cDNA fragments of the desired gene. PCR was performed twice using platinum *Taq* DNA polymerase (Invitrogen) under the following conditions: initial denaturation at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 47°C for 30 s, 68°C for 1 min, with a final extension at 72°C for 10 min. The PCR reaction mixture contained 50 pmol of each primer, and 1 U of DNA polymerase in addition to the template, which was the first strand cDNA for the first PCR and the product of the first PCR amplification for the second PCR. The PCR product was cloned into pT7Blue T-vector and sequenced.

The nucleotide sequence thus obtained was employed to design internal, gene-specific primers that were used for the rapid amplification of cDNA ends (RACE) using a GeneRacer kit (Invitrogen). In brief, the mRNA preparation (250 ng) was treated with calf intestinal phosphatase to dephosphorylate truncated mRNAs, and then treated with tobacco acid pyrophosphatase to remove the 5′-cap structure from the full-length mRNAs. The GeneRacer RNA Oligo was ligated to the 5′-ends of the decapped mRNAs. The ligated mRNA was then reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and a modified lock-docking oligo dT primer, DROTp [5′-GAAGAATTCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTT(A/G/C)-3′]. 5′-End amplification was performed with the GeneRacer 5′-primer and the gene-specific primer 5RFp (5′-AAGCCATGCCTCCTGCGCCTGACTGA-3′) using the PCR setup and cycling parameters suggested by the supplier's protocol. These first amplification products were subjected to a semi-nested PCR using the GeneRacer 5′-primer and the nested gene-specific primer 5RNp (5′-CGCAATAGTTCCCTGGCCAGCAATGAC-3′). The amplification products were gel-purified, ligated into pCR4Blunt-TOPO, and sequenced. 3′-End amplification was performed with the DRAp primer and the gene-specific primer 3RFp (5′-GCTGGCCAGGGAACTATTGCGTTGGA-3′) using the PCR setup and cycling parameters suggested by the supplier's protocol. These first amplification products were subjected to a semi-nested PCR using DRAp primer and the nested gene-specific primer 3RNp (5′-CCAAACCCACCACAGTTCTTAGATAC-3′). The amplification products were gel-purified, ligated into pCR4Blunt-TOPO, and sequenced.

Construction of the pEDR2 Plasmid and Recombinant Aspartate Racemase Expression—A cDNA fragment containing the open reading frame for aspartate racemase was amplified by PCR using Platinum *Pfx* polymerase (Invitrogen) with primers DREFp (5′-TTTCCATATGGCGTCTAAAATTCCACAA-3′) and DRERp (5′-AATTCTCGAGAATTCCTATTTTGTGTCTTTTTTGGTCC-3′), where novel *Nde*I and *Eco*RI sites were introduced at the initiation Met residue and at the stop codons, respectively (underlined sequences), and the total cDNA from *S. broughtonii* as a template. The amplified product was subcloned into a pCR4 Blunt-TOPO vector and the plasmid was double digested with *Nde*I and *Eco*RI; then the excised fragment of DNA was ligated into the corresponding sites of pET25b. The plasmid thus obtained for the expression of the aspartate racemase gene was named pEDR2 and was

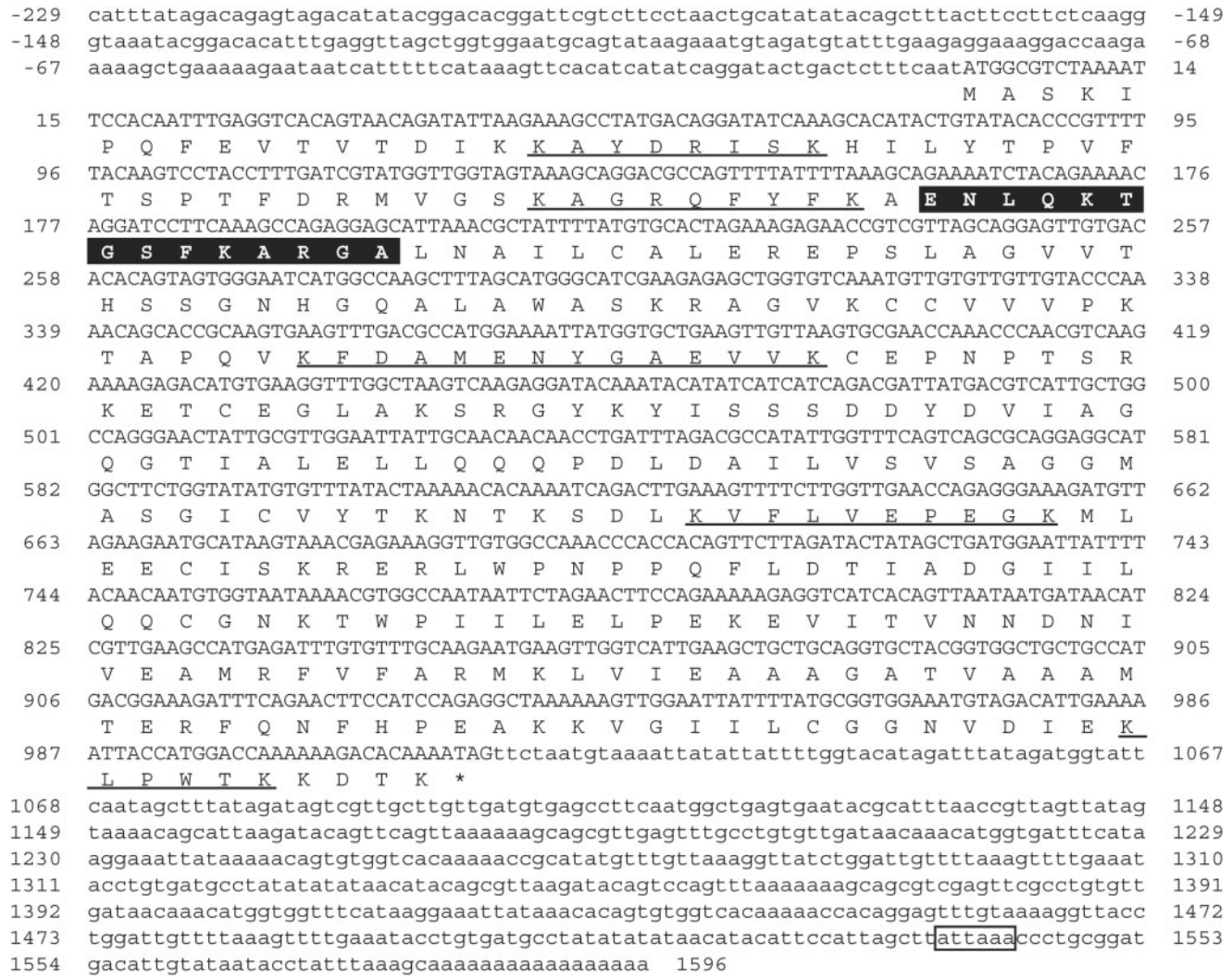

Fig. 1. **Nucleotide sequence of *S. broughtonii* aspartate racemase cDNA and its deduced amino acid sequence.** The numbers on the either side are the nucleotide position from the initiation codon. The coding regions are shown in capital letters, and the non-coding regions are in lower case letters. The asterisk below the nucleotide sequence indicates the termination codon. Amino acid sequences identical to those from internal amino acid sequence analysis (see "MATERIALS AND METHODS") are underlined. A PLP-binding motif (Prosite PS00165) is shown in reverse letters. The putative polyadenylation signal is indicated by the box.

introduced into *E. coli* BL21 (DE3). Transformant cells were grown in LB medium (100 ml) containing 100 μg/ml ampicillin at 30°C. When the absorbance at 600 nm reached 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 mM and the cultures were grown for further 6 h at 30°C, after which the cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C.

Purification of Recombinant Aspartate Racemase—All procedures were carried out at 4°C unless otherwise stated. The enzyme solution was concentrated using an Amicon Diaflo Stirred Cell apparatus with a YM-10 membrane or Centricon-10. Column chromatography was performed on a Shimadzu HPLC system consisting of a Model LC-10Ai pump, a Model SPD-10AV UV-VIS spectrophotometric detector, a Model FCV-10AL low-pressure gradient unit, a Model C-R5A chromatopac integrator, and an appropriate column. The harvested cells were washed once with Buffer A (5 mM Tris-HCl buffer, pH 8.8 containing 1 mM malonic acid, 1 mM EDTA, 2 mM 2-mercaptoethanol and 20 μM PLP) and suspended in the same buffer (10 ml). The cells were homogenized by sonication with a Tomy (Tokyo, Japan) sonic disruptor and the homogenate was centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was filtered through a Minisart filter unit (pore size,0.2 μm) and the filtrate was applied to a column (2.6 × 7.5 cm) of Blue Sepharose 6 FF. After washing the column with the same buffer (820 ml), the enzyme was eluted with a linear gradient from 0 to 500 mM NaCl in the buffer, and active fractions were combined and concentrated. The concentrate (2 ml) was applied to HPLC on a column of HiPrep Sephacryl S-100 HR (1.6 × 60 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.8 containing 1 mM malonic acid, 1 mM EDTA, 2 mM 2-mercaptoethanol, 20 μM PLP, and 150 mM NaCl and the enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min. Malonic acid, which was found to be an inhibitor of the enzyme (data not shown), was included in the buffer in expectation that

it would stabilize the enzyme by binding to the substrate site. Active fractions in the eluate were combined and concentrated.

Production of Antiserum and Western Blot Analysis—Polyclonal antiserum against the recombinant aspartate racemase was raised in a male Japanese white rabbit. The animal was subcutaneously injected with 1 mg of the recombinant enzyme emulsified in Freund's complete adjuvant followed by five subsequent injections, once every 2 weeks, of the same antigen in incomplete Freund's adjuvant. The antiserum thus obtained was used as the primary antibody for Western blot analysis at a 1:3,000 dilution, together with alkaline phosphatase–conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA) as the secondary antibody and an Immuno Blot Kit (Bio-Rad). Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad) by electroblotting before incubation with the antiserum.

Enzyme Assays—Racemization activity was assayed in Buffer B (50 mM Tris-HCl buffer, pH 8.0, containing 1 mM 2-mercaptoethanol and 20 μM PLP) containing the recombinant enzyme (20–1,000 ng) and 300 mM D-aspartate, L-aspartate or any other amino acid tested as a substrate, and other components when required in a final volume of 100 μl. The concentrations of D- and L-aspartate were varied when the kinetic parameters were determined, and temperature and pH were also varied when their effects were examined. The reaction was started by the addition of substrate and stopped after 90 min at 30°C by the addition of 400 μl methanol. Controls were carried out without enzyme. Both D- and L-aspartate, as well as other additional amino acids when required, were determined after derivatization with *o*-phthaldialdehyde and *N*-acetyl-L-cysteine by HPLC as previously described (*12*).

Dehydratase activity was mainly assayed by a colorimetric method depending on the detection of keto acids with 2,4-dinitrophenylhydrazine as follows: Reaction mixtures contained L-aspartate, L-serine, D-serine, L-threonine or L-*threo*-3-hydroxyaspartate at a concentration of 20 mM or 200 mM, and the recombinant aspartate racemase (400 ng) in Buffer B in a total volume of 150 μl. The reaction was started by the addition of substrate and stopped by the addition of 100 μl of 25% trichloroacetic acid after 60 min at 30°C. Controls were carried out without enzyme. After the addition of 100 μl of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl, the mixture was incubated for 15 min at 30°C. Then, 750 μl of 3.75 M NaOH was added to the mixture, which was further incubated for 15 min at 37°C. After centrifugation at 8,700 × *g* for 10 min to remove precipitates, the absorbance of 2,4-dinitrophenylhydrazone was measured at 445 nm. Pyruvate was used as a standard for the reaction of L-serine and D-serine, and oxaloacetate was used for the reaction of L-*threo*-3-hydroxyaspartate.

A spectrophotometric method depending on malate dehydrogenase was further employed to confirm the oxaloacetate production from L-*threo*-3-hydroxyaspartate as follows: Reaction mixtures containing 20 mM L-*threo*-3-hydroxyaspartate, 0.4 mM NADH and 1 unit of malate dehydrogenase in Buffer B (500 μl) were incubated for 3 min at 30°C, and then the reaction was started by the addition of 1 μg of the recombinant aspartate racemase in 10 μl Buffer B. The decrease in NADH was measured over time at 30°C by following the absorbance at 340 nm with a Shimadzu spectrophotometer UV2500-PC equipped with a cell positioner CPS-240A.

One unit of enzyme activity (U) was defined as the amount of enzyme that racemizes or dehydrates 1 μmol of amino acid per minute.

Bioinformatic Analysis—Amino acid sequences were obtained from Swiss-Prot from the ExPaSy proteomics server (http://kr.expasy.org/) of the Swiss Institute of Bioinformatics. A homology search was performed with the FASTA program (*19*) at DDBJ (http://www.ddbj.nig.ac.jp/search/fasta-j.html). The amino acid sequence alignment was performed with Clustal X (*20*). The phylogenetic tree was constructed with N-J Tree (*21*) and drawn with TreeView1.66 (*22*) (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Other Analytical Methods—All DNA sequencing was carried out at least twice on both strands with ALF express II DNA sequencer (Amersham Bioscience) using a Thermo Sequenase cycle sequencing kit with 7-deaza-dGTP and a Thermo Sequenase Cy5 Dye Terminator Kit. Protein concentration was determined by the method of Lowry *et al.* (*23*) using bovine serum albumin as a standard. SDS-PAGE was performed according to Laemmli (*24*). The native molecular mass of the purified recombinant aspartate racemase was analyzed with an HPLC system (Shimadzu) using a Sephacryl S-100 column (1.6 × 60 cm). The column was equilibrated and operated at a flow rate of 0.3 ml/min with a 20 mM Tris-HCl Buffer (pH 7.0) containing 0.2 M NaCl, 20 μM PLP, 1 mM malonic acid, 1 mM EDTA and 2 mM 2-mercaptoethanol. The protein standards used were ribonuclease A, chymotrypsinogen, ovalbumin and albumin from Pharmacia. Absorption spectra were measured with a Shimadzu UV-3100 spectrophotometer.

RESULTS

Nucleotide Sequence of S. broughtonii Aspartate Racemase cDNA—We obtained amino acid sequences for five peptides, included in Fig. 1, from aspartate racemase purified from the foot muscle of the bivalve, and a pair of degenerate primers was synthesized based on two of them. Using these primers and the total cDNA synthesized from the total RNA of the same tissue, a 300 bp cDNA fragment of the enzyme was isolated by PCR. Based on the sequence information in this cDNA fragment, specific primers for the enzyme were synthesized and used for RACE-PCR to obtain the 5′- and 3′-cDNA ends. The entire cDNA sequence thus constructed from these overlapping fragments is shown in Fig. 1 as well as the deduced amino acid sequence. The cDNA is 1,808 bp long and includes an open reading frame of 1,107 bp nucleotides with a polyadenylation signal (ATTAAA) close to the poly(A) tail (nucleotide 1538–1543). The open reading frame encodes a protein of 338 amino acid residues with a predicted molecular mass of 37.1 kDa, in good agreement with the 39 kDa determined for the native enzyme (*12*). It is also confirmed that the deduced amino acid sequence includes sequences identical to those of the five internal peptides obtained from the native enzyme. Moreover, it contains a highly conserved pyridoxal-5′-phosphate binding motif that is also found in the serine/threonine dehydratase family of proteins, consistent with the PLP dependence of the native enzyme.

FASTA searches have revealed that human serine racemase displays the highest overall amino acid sequence identity of 44% to *S. broughtonii* aspartate racemase, followed by 43% identity displayed by mouse and rat serine racemases. The next highest identity is exhibited mainly by threonine dehydratase and its putative homologues of various origin such as *Caenorhabditis elegans* (39%), *Arabidopsis thaliana* (37%), *Xanthomonas axonopodis* (38%) and *Escherichia coli* (33%), in addition to a putative serine racemase from *Saccharomyces cerevisiae* (39%). None of the microbial aspartate racemases show significant identity.

Figure 2 shows multiple alignments of the amino acid sequence of *S. broughtonii* aspartate racemase with those of mammalian serine racemases and biosynthetic threonine dehydratase whose crystal structure has been reported (*25*). In addition to the conserved sequence around the lysine residue to bind PLP through a Schiff

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Mouse SR        ---MCAQYCISFADVEKAHINIQDSIHLTPVLTSSILNQIAG----RNLFFKCELFQKTG 53
Rat   SR        ---MCAQYCISFADVEKAHLNIQDSVHLTPVLTSSILNQIAG----RNLFFKCELFQKTG 53
Human SR        ---MCAQYCISFADVEKAHINIRDSIHLTPVLTSSILNQLTG----RNLFFKCELFQKTG 53
Sb  AspR        MASKIPQFEVTVTDIKKAYDRISKHILYTPVFTSPTFDRMVGSKAGRQFYFKAENLQKTG 60
Eco  TDH        -MADSQPLSGAPEGAEYLRAVLRAPVYEAAQVTPLQKMEKLSSRLDNVILVKREDRQPVH 59
                          :  . :     :   :  :. .*.    .  .    . : .* *  * .

Mouse SR        SFKIRGALNAIRGLIPDTPEEKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQTAPNC 113
Rat   SR        SFKIRGALNAIRGLIPDTLEGKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQTAPNC 113
Human SR        SFKIRGALNAVRSLVPDALERKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQTAPDC 113
Sb  AspR        SFKARGALNAILCALER--EPSLAGVVTHSSGNHGQALAWASKRAGVKCCVVVPKTAPQV 118
Eco  TDH        SFKLRGAYAMMAGLTEE---QKAHGVITASAGNHAQGVAFSSARLGVKALIVMPTATADI 116
                *** ***   :          .  .*:* *:***.*.::::::   *: . :*:* ::.:

Mouse SR        KKLAIQAYGASIVYCDPSDESREKVTQRIMQETEGILVHPNQEPAVIAGQGTIALEVLNQ 173
Rat   SR        KKLAIQAYGASIVYSEPSDESRENVAQRIIQETEGILVHPNQEPAVIAGQGTIALEVLNQ 173
Human SR        KKLAIQAYGASIVYCEPSDESRENVAKRVTEETEGIMVHPNQEPAVIAGQGTIALEVLNQ 173
Sb  AspR        KFDAMENYGAEVVKCEPNPTSRKETCEGLAKSRGYKYISSSDDYDVIAGQGTIALELLQQ 178
Eco  TDH        KVDAVRGFGGEVLLHGANFDEAKAKAIELSQQQGFTWVPPFDHPMVIAGQGTLALELLQQ 176
                *  *:. :*..::   ..  . :    : :.    : . :.  *******:***:*:*

Mouse SR        VPLVDALVVPVGGGGMVAGIAITIKALKPSVKVYAAEPSN--ADDCYQSKLKGELTPN-L 230
Rat   SR        VPLVDALVVPVGGGGMVAGIAITIKTLKPSVKVYAAEPSN--ADDCYQSKLKGELTPN-L 230
Human SR        VPLVDALVVPVGGGGMLAGIAITVKALKPSVKVYAAEPSN--ADDCYQSKLKGKLMPN-L 230
Sb  AspR        QPDLDAILVSVSAGGMASGICVYTKNTKSDLKVFLVEPEGKMLEECISKRER--LWPNPP 236
Eco  TDH        DAHLDRVFVPVGGGGLAAGVAVLIKQLMPQIKVIAVEAED---SACLKAALDAGHPVD-L 232
                 . :* :.*.*..**: :*:.:  *   ..:**  .*...   . * .         :

Mouse SR        HPPETIADG-VKSSIGLNTWPIIRDLVD-DVFTVTEDEIKYATQLVWGRMKLLIEPTAGV 288
Rat   SR        HPPETIADG-VKSSIGLNTWPIIRDLVD-DVFTVTEDEIKYATQLVWERMKLLIEPTAGV 288
Human SR        YPPETIADG-VKSSIGLNTWPIIRDLVD-DIFTVTEDEIKCATQLVWERMKLLIEPTAGV 288
Sb  AspR        QFLDTIADGIILQQCGNKTWPIILELPEKEVITVNNDNIVEAMRFVFARMKLVIEAAAGA 296
Eco  TDH        PRVGLFAEGVAVKRIGDETFRLCQEYLD-DIITVDSDAICAAMKDLFEDVRAVAEPSGAL 291
                     :*:*   .  * :*: :  :  : :::** .* *  * : ::  :: : *.:..

Mouse SR        ALAAVLSQHFQTVSPEVKNVCIVLSGGNVDLTS-LNWVGQAERPAPYQTVSV-------- 339
Rat   SR        GLAAVLSQHFQTVSPEVKNICIVLSGGNVDLTS-LSWVKQAERPAP-------------- 333
Human SR        GVAAVLSQHFQTVSPEVKNICIVLSGGNVDLTSSITWVKQAERPASYQSVSV-------- 340
Sb  AspR        TVAAAMTERFQNFHPEAKKVGIILCGGNVDIEK-LPWTKKDTK----------------- 338
Eco  TDH        ALAG-MKKYIALHNIRGERLAHILSGANVNFHG-LRYVSERCELGEQREALLAVTIP... 514
                 :*. :.: :     . :.:  :*.*.**::   : :. :  .
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Fig. 2. **Multiple alignment of the amino acid sequence of *S. broughtonii* aspartate racemase (Sb AspR) with those of mouse, rat and human serine racemase (SR) homologues, and *E. coli* biosynthetic threonine dehydratase (Eco TDH).** The alignment was generated with Clustal X 1.83 (*20*). The numbers on the right side are the residue numbers of each amino acid sequence. Identical residues and amino acid substitutions with low and high similarities are indicated by asterisks, dots, and double dots, respectively. The putative PLP-binding lysine residue is indicated by reversed letters. The amino acid residues predicted to interact with PLP are indicated by gray boxes. A PLP-binding motif is indicated by boxes. Underline indicates the tetraglycine loop. For accession numbers see Fig. 3.

base, other residues that have been shown to interact with PLP in threonine dehydratase (*25*) and that are conserved in all the mammalian serine racemases are mostly conserved in aspartate racemase. The phylogenetic analysis presented in Fig. 3 suggests that *S. broughtonii* aspartate racemase is only distantly related to microbial aspartate racemases, similar to glutamate racemases and alanine racemases, and is rather closely related to threonine dehydratases as well as serine racemases.

Expression of S. broughtonii Aspartate Racemase— A cDNA fragment containing the entire open reading frame of *S. broughtonii* aspartate racemase was isolated by PCR with appropriate primers and the total cDNA, and was inserted into pET25b to generate an expression vector, pEDR2, which was then expressed in *E. coli* BL21 (DE3) in the presence of 1 mM IPTG. The crude cell extract showed significant aspartate racemase activity, whereas far lower activity (15%) was exhibited in extracts of cells not treated with IPTG, and no activity was observed in extracts of cells harboring the control vector, pET25b. The specific activity of the recombinant enzyme (about 1 μmol min^{-1} mg^{-1}) was 130 times of that of the *S. broughtonii* muscle homogenate (*12*), and the enzyme was estimated to represent about 10% of the total bacterial soluble protein by calculation assuming that the recombinant enzyme exhibits the same specific activity as the native enzyme purified from the bivalve (*12*).

On SDS-PAGE analysis, an intense band is apparent in the extract and pellet of the induced cells harboring pEDR2 at a position with a predicted molecular mass (37.1 kDa) of the native aspartate racemase, and this band is not observed in the other lanes. This suggests that the recombinant enzyme in the induced cells is present not only in a soluble active form, but also as insoluble inclusion bodies.

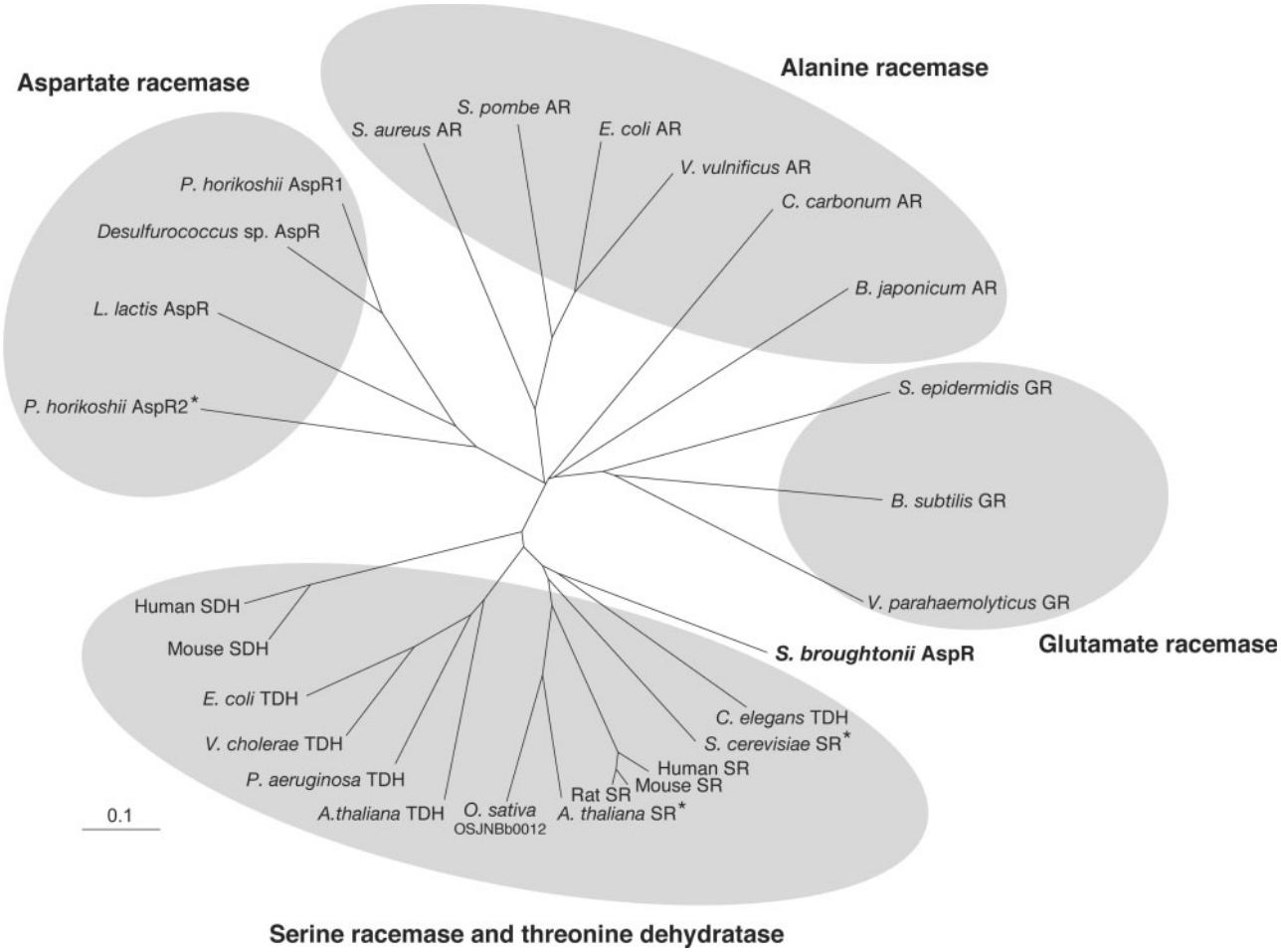

Fig. 3. **Phylogenetic relationships among amino acid racemases and serine/threonine dehydratases from various organisms.** The phylogenetic tree was created with the Clustal X 1.83 (*20*) and TreeView 1.66 (*22*) programs. The scale bar represents 0.1 amino acid substitution per site. The asterisks indicate putative enzymes. Swiss-prot accession number: *Staphylococcus aureus* alanine racemase (AR) Q99SI5; *Schizosaccharomyces pombe* AR O59828; *E. coli* AR Q8FB20; *Vibrio vulnificus* AR Q8DCL0; *Cochliobolus carbonum* AR Q9UW18; *Bradyrhizobium japonicum* AR Q89MX1; *Pyrococcus horikoshii aspartate* racemase (AspR)1 O58403; *Desulfurococcus* sp. AspR P71164; *Lactococcus lactis* AspR Q9CDJ4; *P. horikoshii* AspR2 O59384; *Staphylococcus epidermidis* glutamate racemase (GR) Q5HQ24; *Bacillus subtilis* GR O05412; *Vibrio parahaemolyticus* GR Q87KP1; Human serine dehydratase (SDH) P20132; Mouse SDH Q8VBT2; *E. coli* threonine dehydratase (TDH) P04968; *Vibrio cholerae* TDH Q9KVW1; *Pseudomonas aeeruginosa* TDH Q9I6G0; *Arabidopsis thaliana* TDH Q9ZSS6; *Oryza sativa* OSJNBb0012 (function unknown) Q7XSN8; *Arabidopsis thaliana* putative serine racemase (SR) Q9T0D1; Rat SR Q76EQ0; Mouse SR Q9QZX7; Human SR Q9GZT4; *Saccharomyces cerevisiae* putative SR P36007; *Caenorhabditis elegans* SR Q19329.

Table 1. **Purification of recombinant aspartate racemase from *E. coli* BL21 (DE3) harboring pEDR 2.**[a] A typical example of four independent experiments is given.

Step/Fraction	Protein (mg)	Total activity[b] (U)	Yield (%)	Specific activity (U/mg protein)	Purification (fold)
Homogenate	446	274	100	0.614	1.00
Crude extract	266	289	105	1.09	1.78
Blue Sepharose	6.68	60.2	21.9	9.03	14.7
Sephacryl S-100	2.88	28.8	10.5	10.0	16.3

[a]Starting material was 5.47 g of wet cells from 0.9 liter culture of *E. coli* BL21 (DE3) harboring pEDR2. [b]The activity was measured with 200 mM L-aspartate as substrate and D-aspartate produced was determined by HPLC after derivatization with *o*-phthaldialdehyde and *N*-acetyl-L-cysteine.

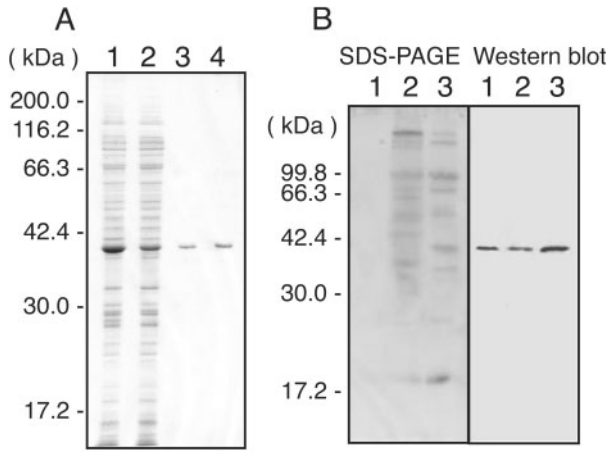

Fig. 4. **SDS-PAGE analysis of samples at the purification stages of recombinant aspartate racemase from *E. coli* cells (A) and Western-blot analysis of partially purified preparations of native aspartate racemase from *S. broughtonii* foot muscle (B).** A: lane 1, homogenate (15 μg); lane 2, crude extract (15 μg); lane 3, Blue Sepharose column fraction (0.5 μg); lane 4, Sephacryl S-100 column fraction (0.5 μg). Proteins were stained with Coomassie Brilliant Blue. B: lane 1, purified recombinant aspartate racemase (0.15 μg); lane 2, 1.2–2.4 M sodium sulfate precipitate (36 μg) of foot muscle extract; lane 3, Blue Sepharose column fraction (2.6 μg) of foot muscle extract. Detection was carried out with antiserum raised against recombinant aspartate racemase as the primary antibody at a 1:3,000 dilution.

Purification and Characterization of Recombinant S. broughtonii Aspartate Racemase—Table 1 summarizes the results of a typical purification procedure. The recombinant aspartate racemase was purified 16.3-fold from the homogenate to a final preparation with a specific activity of 10.0 U/mg, which is nearly equal to that of the native enzyme (9.7 U/mg) (*12*). SDS-PAGE of the final preparation gave a single band with an estimated molecular mass of approximately 38 kDa (Fig. 4A), which is close to the 39 kDa of the native enzyme (*12*) and the 37.1 kDa predicted from the deduced amino acid sequence (Fig. 1). Furthermore, Western blot analysis with polyclonal antiserum raised against the recombinant enzyme detected a single band with the same mobility as the recombinant enzyme (Fig. 4B) in enzyme preparations partially purified from the bivalve. On gel filtration through Sephacryl S-100, the enzyme showed a sharp single peak with an estimated molecular mass of 56 kDa, in agreement with the 51–63 kDa estimated for the native enzyme. The molecular mass thus obtained, in conjunction with the results of SDS-PAGE, suggest a homodimeric enzyme structure, which was not well supported by the previously obtained broad value for the native enzyme. Sequencing of the amino terminal region of the recombinant enzyme up to the 15th amino acid residue confirmed the deduced amino acid sequence shown in Fig. 1, and not the amino acid sequence previously reported for the native enzyme (*12*), posing a question about the discrepancy.

The presence of PLP bound to the enzyme was evident from the absorption spectrum, which exhibited a maximum at 420 nm, and was markedly changed by the addition of amino-oxyacetate (data not shown). The PLP-dependence of the enzyme activity was obvious from the complete inhibition exerted by 10 mM amino-oxyacetate and 10 mM hydroxylamine with several other compounds including phenylhydrazine and L-cycloserine showing a similar pattern of inhibition to that reported for the native enzyme. It was ascertained that EDTA was not inhibitory, just as in the case of the native enzyme. This finding is in contrast to the reported properties of mammalian serine racemase that is markedly inhibited by EDTA and requires divalent cations for full enzyme activity (*16*, *26*).

As shown in Table 2, the enzyme was confirmed to racemize only D- and L-aspartate and not other amino acids. Moreover, it was revealed that the enzyme shows a dehydratase activity toward L-*threo*-3-hydroxyaspartate, producing oxaloacetate that was detected with 2,4-dinitrophenylhydrazine and by the dehydrogenation of NADH catalyzed by malate dehydrogenase. The enzyme seems to exhibit similar activity toward L-serine very slightly, although the product was not further identified.

The effects of nucleotides on the native enzyme were also confirmed for the recombinant enzyme: 10 mM AMP and ADP increased the activity to 399 and 320% of the control, respectively, whereas 10 mM ATP decreased it to 23%. Compared with the findings for the native enzyme that the same concentrations of AMP, ADP and ATP changed the activity to 173, 116 and 23%, respectively (*18*), the recombinant enzyme appears to be more sensitive to AMP and ADP. Optimal pH and temperature of the recombinant enzyme were 8.5 and 37°C, respectively, in comparison with 8.0 and 25°C, respectively (*12*), for the native enzyme. The K_m and V_{max} values were 14.5 mM and 6.20 U mg^{-1} for L-aspartate, and 17.2 mM and 8.10 U mg^{-1} for D-aspartate, respectively, while previously reported K_m and V_{max} values for the native enzyme were 60.4 mM and 7.39 U mg^{-1} for L-aspartate, and 159 mM and 22.6 U mg^{-1} for D-aspartate, respectively (*12*). The K_m values for the recombinant enzyme seem to be lower than those for the native enzyme and, as well as V_{max} values, relatively insensitive to the configuration of the substrate.

Table 2. **Racemase and dehydratase activities of recombinant aspartate racemase.** The reaction was carried out in 50 mM Tris-HCl buffer, pH 8.0 containing 1 mM 2-mercaptoethanol and 20 μM PLP using 20–1,000 ng recombinant enzyme. Racemization products were derivatized with *o*-phthaldialdehyde and *N*-acetyl-L-cysteine and analyzed by HPLC. Dehydration and hydrolysis products were determined colorimetrically after treatment with 2,4-dinitrophenylhydrazine, unless otherwise stated.

Substrate	Relative activity (%)[a]	
	Racemase	Dehydratase
Experiment 1		
300 mM L-Aspartate	100	NT[b]
300 mM L-Glutamate	0	NT
300 mM L-Alanine	0	NT
300 mM L-Serine	0	NT
Experiment 2		
200 mM L-Aspartate	100	0
200 mM L-Serine	NT	0.5 (0.1)[c]
200 mM D-Serine	NT	0
Experiment 3		
20 mM L-Aspartate	100	0
20 mM L-Serine	NT	0.7 (0.0)[c]
20 mM L-*threo*-3-Hydroxyaspartate	NT	16.4 (0.1)[c,d]

[a]Racemase activities toward L-aspartate were 9.97, 7.76 and 4.26 U/mg in experiment 1, 2 and 3, respectively. [b]Not tested. [c]Standard deviations of triplicate determinations are given in parenthesis. [d]Assayed also by the spectrophotometric method with malate dehydrogenase that showed 11.0% relative activity by a single determination.

DISCUSSION

The isolation and sequencing of the complete cDNA coding for a PLP-dependent aspartate racemase of animal origin, as well as the expression of the recombinant active enzyme, are reported here for the first time. The amino acid sequence deduced from the nucleotide sequence displays no significant homology to those of known aspartate and glutamate racemases. This is not surprising, since these racemaces are all PLP-independent and, therefore, considered not to be closely related to the present enzyme. The highest amino acid sequence identity (43–44%) to this enzyme is exhibited by mammalian serine racemase, which represents the first animal amino acid racemase whose primary structure was clarified, and the next highest identity is shown by a putative serine racemase, threonine dehydratase and its putative homologues of various organisms including plant, yeast and bacteria. Moreover, the amino acid residues known to interact with PLP in *E. coli* biosynthetic threonine dehydratase and conserved in the mammalian racemase are mostly conserved in the present aspartate racemase as well. Thus the present aspartate racemase, as well as the mammalian serine racemase, seems to be related to the fold-type II group of PLP enzymes, such as serine/threonine dehydratase. Alanine racemase from the kuruma prawn *Penaeous japonicus* is one of the small number of animal amino acid racemases whose cDNAs have been isolated, but the deduced amino acid sequence shows, at most, 27% homology with that of bacterial and yeast (*27*) enzymes, suggesting that this crustacean enzyme is not closely related to the bivalve aspartate racemase nor to the mammalian serine racemase.

The recombinant aspartate racemase is essentially identical to the native enzyme in its behavior on SDS-PAGE and gel filtration, in possessing a bound PLP and strict specificity for aspartate as a substrate of racemization, and in its sensitivity to adenine nucleotides and to various inhibitors. In addition, immunochemical identity was confirmed between the native enzyme in the partially purified preparations from bivalve tissue and the recombinant enzyme by Western blot analysis with antiserum raised against the recombinant enzyme.

The observed activity toward L-*threo*-3-hydroxyaspartate is a new finding that was not made with the minute amount of native enzyme available, and could be a new example of the activity detected in eukaryotes. This activity may represent only a side-reaction often observed for PLP-dependent enzymes due to the chemistry of PLP. However, it is also possible that the activity somehow reflects the structural homology between this enzyme and threonine dehydratases as well as serine racemase that also show dehydratase activity (*16*, *28*). This possibility appears to be enhanced when it is considered that the serine racemase homologue of *Saccharomyces cerevisiae*, the product of the SRY1 gene, turned out to be L-*threo*-3-hydroxyaspartate dehydratase (*29*). As shown in Table 2, there was no indication that L-aspartate is a substrate for the dehydratase activity of the aspartate racemase. This would mean that possession of a hydroxyl group is required for the substrate. In this sense, the data in Table 2 also suggest that L-serine behaves as a poor substrate because this neutral amino acid with a hydroxyl group little binds to this aspartate-specific enzyme. Recently, we learned that L-threo-3-hydroxyaspartate behaves as an excellent substrate, much better than D- and L-serine, for the dehydratase activity of mouse serine racemase, as well as that L-*erythro*-3-hydroxyaspartate represents the most effective inhibitor of the same enzyme (*30*). These findings, no doubt, indicate a closer functional similarity between serine racemase and aspartate racemase, although many more structural and kinetic studies are required to understand the significance of the dehydratase activity of aspartate racemase.

One of the characteristic properties found for native aspartate racemase and confirmed with the recombinant enzyme is its sensitivity to nucleotides: the activity is increased by AMP and decreased by ATP, in contrast to serine racemase whose activity is oppositely increased by ATP. ADP also increases the activity, although less effectively than AMP. In this respect, the present enzyme resembles the biodegradative threonine dehydratase of *Salmonella typhimurium*, which is similarly activated by AMP and ADP (*31*). This pattern may reflect the roles of these enzymes in energy metabolism, since aspartate racemase is probably involved in the utilization of D-aspartate as a substrate for anaerobic energy metabolism in *S. broughtonii* (*32*), while serine racemase is unlikely to contribute to energy metabolism significantly (*17*). It would be of interest to clarify and compare the binding sites for these nucleotides.

There appear to be some functional and structural differences between the recombinant and native enzyme previously reported, for example, in the K_m values and the

amino acid sequence in the amino terminal region. Functional differences would be also based on some variations in the structure. As for the structural differences, it is possible that the native enzyme contains attached sugar chains and/or phosphorylated residues, although the very small amount of native enzyme available did not allow us to examine these possibilities. In this regard, a consensus pattern for the *N*-glycosylation site, N-X-S/T, is observed in the amino acid sequence of the recombinant enzyme (253–255) given in Fig. 2. It is also possible that isoforms of the enzyme are present as shown for serine racemase (*33*). In addition, various cases of polymorphism due to molecular microheterogeneity and proteolysis during purification are discussed, for instance for D-aspartate oxidase of bovine kidney (*34*) and its recombinant form (*35*). It has been also pointed out that the possibility of alternate splicing in higher eukaryotes can increase the functional diversity of enzymes (*36*). At present, we are not ready to propose any plausible hypothesis about the stuructural differences, even with these raised possiblities. To obtain clues to the question, much larger amounts of enzyme must be prepared than those reported (*12*) from bivalve tissues, and, prior to that, more efficient procedures for purification of the enzyme need to be developed. While these tasks remain to be carried out, the present results demonstrate that the bivalve aspartate racemase is homologous to mammalian serine racemase, and this will facilitate the discovery of a mammalian aspartate racemase whose presence has been strongly suggested to support the production of D-aspartate present in mammalian tissues.

The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AB231327

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