Gene for Aspartate Racemase from the Sulfur-dependent Hyperthermophilic Archaeum, Desulfurococcus Strain SY*

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Amino acid racemases are ubiquitous throughout eu­bacteria. However, no amino acid racemases have yet been found in eukaryotes and archaea. We cloned a gene highly homologous to that for the aspartate racemase from the sulfur-dependent hyperthermophilic archaeum, Desulfurococcus strain SY. The product of the gene showed 35.2% amino acid sequence identity with the aspartate racemase of Streptococcus thermophilus IAM10064, and was also homologous to glutamate racemases around the putative catalytic cysteine residues. The encoded protein was expressed in Escherichia coli. The recombinant protein had amino acid racemizing activity, which was highly specific for aspartate and increased with temperature from 37 °C to 90 °C. Therefore, this was identified as the first hyperthermophilic archaenal amino acid racemase. A little aspartate racemizing activity was also detected in the crude extract of Desulfurococcus strain SY. The function of this aspartate racemase might be the uptake of D-aspartate as a cell component. The fact that the amino acid racemases are distributed among both eubacteria and archaea suggests that endogenous D-amino acids in mammals are also synthesized by amino acid racemases.

D-amino acids are important components of eubacteria, as they constitute parts of the fundamental tetrapeptide chain in murein of the cell wall (1). D-Alanine is formed from L-alanine by alanine racemase (EC 5.1.1.1) which is quite widespread in eu­bacteria (2). Other D-amino acids, such as D-aspartate or D-glutamate, are formed either by transamination from L-alanine to the appropriate α-keto acids or by racemization of the enantiomeric L-amino acids (3–7). Alanine racemase requires pyridoxal 5'-phosphate as a cofactor. Racemization proceeds through the formation of an aldime Schiff base between the substrate alanine and pyridoxal 5'-phosphate (8). However, several other amino acid racemases, including those for glutamate and aspartate, are cofactor independent (6, 7, 9–12). In these racemases, thiol groups of cysteine residues serve as catalytic bases in the proton transfer reactions.

There are few reports describing endogenous D-amino acids in eukaryotes or archaea. Although it is proposed that the biosynthesis of D-serine in worms and insects involves amino acid racemases (13), these enzymes have not been found in vertebrates or mammals. Several groups have identified D-serine, D-aspartate, and D-glutamate in mammals, including humans (14–16). The biosynthetic pathways of these D-amino acids remain unknown.

Murein is the only cell wall polymer that forms rigid cell walls in eu­bacteria. However, archaea have a variety of cell walls and cell envelope polymers (17, 18). Many archaea (including all crenarchaeots, euryarchaeots, halophiles, and methanogens) have an outer envelope (or S-layer) composed of hexagonally or tetragonally arranged proteins or glycoproteins that are easily disintegrated by mechanical shearing or detergents. In methanogens, the structure of this polymer is similar to that of murein, and it is called "pseudomurein." Pseudomurein differs from murein in several respects. No D-amino acids are present in pseudomurein. Thus, neither D-amino acids nor aspartic acid racemases have been found in archaea.

Hyperthermophiles which grow optimally at over 80 °C have been isolated (19, 20). Most of them are archaea. Since the hyperthermophiles are located in deep branches close to the root of the phylogenetic tree based on 16/18S rRNA (21), it is suggested that hyperthermophiles retain some of the physiological or biochemical features of early life forms. On the other hand, they produce highly thermostable enzymes which are industrially useful.

Here, we describe the cloning of the gene encoding aspartate racemase from the hyperthermophilic archaeum, Desulfurococcus strain SY (22), which was inadvertently cloned during the search for homologues of hsp70 stress proteins in hyperthermophilic archaea. This is the first archaenal amino acid racemase described to date.

**EXPERIMENTAL PROCEDURES**

Reagents—Restriction and modification enzymes were the products of Takara Shuzo Co., Ltd. Taq DNA polymerase and other reagents for the polymerase chain reaction (PCR) were also obtained from Takara Shuzo Co., Ltd. Nylon membranes and the ECL random primelabeling and detection system were from Amersham. D-Amino acid oxidase from pig kidney was the product of Biozyme Laboratories, Ltd. Horseradish peroxidase was purchased from Wako Pure Chemical Industries, Ltd. All other chemicals were of analytical grade.

Bacterial Strain, Cultivation, and DNA Extraction—All plasmids were propagated in Escherichia coli DH5α (Life Technologies, Inc.). The recombinant aspartate racemase was expressed in E. coli BL21(DE3) (23). E. coli was cultured aerobically at 37 °C in 2× YT media supple-

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¶The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; IPTG, isopropl-1-thio-β-D-galactopyranoside; hsp70, heat shock protein 70; D SY, Desulfurococcus strain SY.
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An expression system for the aspartate racemase of an oligonucleotide primer (5’-AGG-AAT-CTA-TAT-TGC-GCA-GA-3’) was designed to generate an NdeI digestion site at the initiation codon. It also contains an EcoRI site at the 5’ terminal for subcloning. Another primer (5’-TT-AAG-GGC-GGC-AGT-GTA-3’) corresponding to the region immediately downstream of the PstI site in the coding sequence was synthesized. Using the primer set, a 432-bp DNA fragment of pAB101, corresponding to the 5’-terminal half of the coding sequence was amplified by PCR (30 cycles of 60 s at 95°C, 30 s at 50°C, and 120 s at 72°C) from the plasmid, pAB101. The amplified fragment was digested with EcoRI and PstI, subcloned into pUC18, and sequenced. Among several clones sequenced, pAB501, which was free from mutations, was selected and further manipulated. The total DNA was digested with restriction enzymes that were predicted to cleave the entire aspartate racemase gene, hybridized to the identified DNA fragment as a probe. HindIII was selected because a 344-bp PstI/HindIII fragment of pAB101, corresponding to the 3’-terminal of the open reading frame in the clone, as a probe. The cloned pAB401 contained about 1.8 kilobase pair of PstI/HindIII fragment covering the 3’-terminal of the gene (Fig. 1A).

The sequence of a 1440-bp DNA region including a 705-bp open reading frame, encoding a 235-residue polypeptide, is shown in Fig. 1B. The underlines indicate the binding sites of the PCR primers used for the screening. Although a ribosomal binding sequence (Shine-Dalgarno sequence) was found just before the initiation codon, no putative archaean promoter sequence was found. The estimated molecular weight of the encoded protein was 25,977. It shared considerable homology (35.2% identity and 63.1% similarity in amino acid sequence) with the aspartate racemase of the lactic eubacterium, *S. thermophilus* (31) (Fig. 2A).

The amino acid sequence was also homologous to those of glutamate racemases from *Bacillus sphaericus*,2 *E. coli* (32, 33), *Lactobacillus brevis* (34), *Lactobacillus fermenti* (35), and *Pediococcus pentosaceus* (36). The homology scores were 28.2, 24.0, 21.6, 27.6, and 27.7%, respectively.

Two cysteine residues, which are thought to be the catalytic centers of these cofactor independent racemases, as well as the surrounding amino acid sequences, were highly conserved among these enzymes (Fig. 2B).

Expression of the Aspartate Racemase in *E. coli*—To confirm that the cloned gene really encodes the aspartate racemase, the gene was expressed in *E. coli* using the T7 polymerase expression system (23). The entire open reading frame of the gene was subcloned into the expression vector pET21c, yielding pAB503 (Fig. 3A). The reading frame was preceded by an *E. coli* ribosomal binding site under the control of the viral T7 promoter. *E. coli* strain BL21(DE3), which expresses T7 polymerase by the induction of IPTG, was transformed with pAB503. The transformant expressed a protein of about 26 kDa (Fig. 3B) 6 h

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1. *GenBank* data submitted by I. G. Fotheringham, S. A. Bleigid, and P. P. Taylor under accession number U26733.
after adding 2 mM IPTG. The molecular weight coincided well with the value predicted from the nucleotide sequence. The amount of the expressed protein was about 10% of the total protein in *E. coli*. The expressed racemase was soluble and remained stable after an incubation at 70°C for 30 min (Fig. 3C). The purity of the aspartate racemase was estimated to be more than 95%. Thus, this procedure would be very effective for purifying this hyperthermophilic aspartate racemase.

**Amino Acid Racemase Activity**—The amino acid racemase activity of the heated cell-free extract of *E. coli* BL21(DE3) harboring pAB503 was measured, using alanine, aspartate, and glutamate as the substrates (inset of Fig. 4). At 37°C, the extract showed a little aspartate racemase activity. Since the *E. coli* alanine racemase was heat-denatured, this activity was absent. At 70°C, the extract showed high aspartate racemase activity. However, neither alanine nor glutamate was racemized even at this temperature. The racemization activity of alanine or glutamate at 70°C was none or at most 0.3% of that of aspartate racemase. These results showed that the encoded protein is a thermophilic amino acid racemase that is highly specific for aspartate.

**Fig. 1.** Restriction map, nucleotide, and predicted amino acid sequences of the aspartate racemase gene. A, the open reading frame encoding aspartate racemase is shown by a hatched box with an arrow. B, nucleotide sequence and predicted amino acid sequence. The upper line shows the nucleotide sequence, and the second line shows the predicted amino acid sequence. The underlines indicate the binding sites of the PCR primers used for screening. The putative ribosome binding sequence is marked by a double underline. The presumed catalytic and conserved cysteine residues are shaded.
from 37°C to 90°C as shown in Fig. 4. This result confirmed that the gene originated from a hyperthermophile.

To confirm that D. SY really expresses the gene, we measured the aspartate racemase activity in the crude extract of D. SY. Aspartate racemase activity in the extract was about 0.004 unit/mg of protein at 70°C.

**DISCUSSION**

Aspartate racemases have been found only in lactic acid bacteria (7). Staudenbauer and Strominger (37) have described the incorporation of L-aspartic acid into murein in *S. faecalis* and *L. casei* (37). Only the aspartate racemase of the lactic acid bacterium, *S. thermophilus*, had been purified and cloned (7, 31). Although D. SY is evolutionarily distinct from eubacteria, the aspartate racemase of D. SY was highly homologous to that of *S. thermophilus* (Fig. 2A). Both of these aspartate racemases are highly related to glutamate racemases (Fig. 2B).

Several amino acid racemases, including proline racemase, diaminopimelate epimerase, glutamate racemase, and aspartate racemase, are cofactor independent and use thiol groups of cysteine residues as bases (6, 9, 10, 12, 28). Amino acid racemization proceeds by means of one- and two-base mechanisms. The reaction by pyridoxal 5'-phosphate-dependent amino acid racemases proceeds by the one-base mechanism (8). Cofactor independent amino acid racemases achieve racemization by the two-base mechanism (9, 10, 12, 28, 38). In the latter, an α-hydrogen of an amino acid is abstracted on one face as a proton while a proton is incorporated on the other face. Thus, two cysteine residues are required for the reaction by these amino acid racemases. Proline racemase and aspartate racemase consist of two identical subunits. It is likely that the active sites of these racemases are formed at the interface of two identical subunits, each of which provides one catalytic cysteine residue. However, glutamate racemase exits as monomer. Thus, the two catalytic cysteine residues are in one subunit (38). The cysteine residues and surrounding amino acid sequences are highly conserved among all cloned and sequenced aspartate and glutamate racemases (Fig. 2B).

Although Cys-197 of the *S. thermophilus* aspartate racemase is not essential for catalysis (28), the cysteine residue and surrounding amino acid sequence are also conserved in D. SY aspartate racemase. The regions surrounding the conserved core sequences of aspartate racemases are quite distinct from those of glutamate racemases. Since both aspartate racemases from *S. thermophilus* and D. SY are highly specific for aspartate, the regions around the conserved core sequences might be related to substrate specificity.

The recombinant D. SY aspartate racemase expressed in *E. coli* was extremely stable (Fig. 4). This enzyme would be useful for studying the structure and function of cofactor-independent amino acid racemases.

It has been believed that archaea contain neither L-amino acids nor amino acid racemases. However, the results of this study refute this notion. The functions of the aspartate racemase and the produced α-aspartate in D. SY are of interest. This hyperthermophile is an obligate heterotroph that requires amino acids for growth (39). Although most amino acids spontaneously racemize very slowly, aspartate does so rapidly at...
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The nucleotide sequence showed that one of the oligonucleotides used for PCR hybridized to the noncoding region. This means that the gene was amplified by chance and that there is no functional or structural relationship between this aspartate racemase and hsp70s.

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