

Occurrence of Free D-Amino Acids and Aspartate Racemases in Hyperthermophilic Archaea

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The occurrence of free D-amino acids and aspartate racemases in several hyperthermophilic archaea was investigated. Aspartic acid in all the hyperthermophilic archaea was highly racemized. The ratio of D-aspartic acid to total aspartic acid was in the range of 43.0 to 49.1%. The crude extracts of the hyperthermophiles exhibited aspartate racemase activity at 70°C, and aspartate racemase homologous genes in them were identified by PCR. D-Enantiomers of other amino acids (alanine, leucine, phenylalanine, and lysine) in *Thermococcus* strains were also detected. Some of them might be by-products of aspartate racemase. It is proven that D-amino acids are produced in some hyperthermophilic archaea, although their function is unknown.

D-Amino acids are important components of eubacteria, as they constitute the peptide chains in the murein of cell walls (23). However, there are a few reports describing endogenous D-amino acids in *Eucarya* or *Archaea*. Free D-serine and D-aspartic acid in mammals have been identified by several groups (3, 7). D-Serine in the silkworm, *Bombyx mori*, is also known to occur. Very recently, pyridoxal 5'-phosphate-dependent serine racemases in the silkworm and in rat brain were found (22, 24).

Archaea possess a broad range of cell envelope structural formats, but murein has not been found in their cell walls or envelopes (12, 13). Cell envelopes of some archaea, including methanogens, consist of pseudomurein whose structure is homologous to murein. However, pseudomurein does not contain D-amino acids in its peptide unit (14). Thus, it was believed that there were neither D-amino acids nor amino acid racemases in *Archaea*. However, a gene encoding aspartate racemase in the sulfur-dependent hyperthermophilic archaeum *Desulfurococcus* sp. strain SY, has been found (27). Aspartate racemase activity in the crude extract of the strain has also been detected (27). Recently, total genomic sequences of several archaea have been revealed (2, 15–17, 19). Among them, homologues of the aspartate racemase gene in *Archaeoglobus fulgidus* and *Pyrococcus horikoshii* OT3 were identified. The occurrence of peptidyl D-amino acids in several archaea was also reported (18). Thus, it is suggested that D-amino acids and amino acid racemases are widely distributed and function in archaea. This report describes the distribution of aspartate racemases and free D-amino acids in some hyperthermophilic archaea, such as *Thermococcus* and *Pyrococcus* strains.

Free D-amino acids in hyperthermophilic archaea. The aspartate racemase gene in the hyperthermophilic archaeum

Desulfurococcus sp. strain SY has been detected and aspartate racemase activity in the same strain has also been found (27). However, the function of the aspartate racemase is unknown. Then we determined the amount of free D-amino acids in several hyperthermophilic archaea, including *Desulfurococcus* sp. strain SY (27).

The hyperthermophilic archaea *Desulfurococcus* sp. strain SY (10), *Thermococcus* sp. strains KS-1, KS-8, and KI (8), and *Pyrococcus* sp. strains GB-D (11) and OII, which had been isolated from a coastal hot spring on Iwo Jima Island, Japan, were cultured at 90°C in 5-liter glass bottles as described previously (9). The cells were collected by centrifugation at 10,000 × g for 15 min at 10°C and used in this study.

The content of free D-amino acids was determined as described previously, with slight modification (6). The frozen cells were homogenized in 10 volumes of 0.25 M NaCl at room temperature. To remove protein fractions and extract amino acids, the homogenate was further homogenized after the addition of 10 volumes of methanol. The homogenate was centrifuged at 7,000 × g for 5 min, and 50 μl of the resultant supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 20 μl of 50 mM borate buffer (pH 8.0), and 10 μl of water and 30 μl of 20 mM NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole), a fluorogenic derivatizing reagent, in acetonitrile was added to the solution. The reaction mixture was heated at 60°C for 2 min and was mixed with 440 μl of 1% trifluoroacetic acid. After being filtered through a 0.5-μm membrane filter (column guard LCR4; Millipore), the sample was analyzed for NBD-F-derivatized amino acids (6). Each amino acid derivatized with NBD-F was isolated and quantified fluorometrically as the sum of L and D isomers by reverse-phase high-pressure liquid chromatography (HPLC) with an octyldecyl silane column (J-sphere ODS-M80). The fraction which contained the L and D isomers was evaporated to dryness under reduced pressure and the residue was dissolved with 1% acetic acid in methanol. Subsequently, enantiomers of the amino acids were separated by HPLC with a Pirkle-type chiral column (Sumichiral OA2500[S] or -[R]) and

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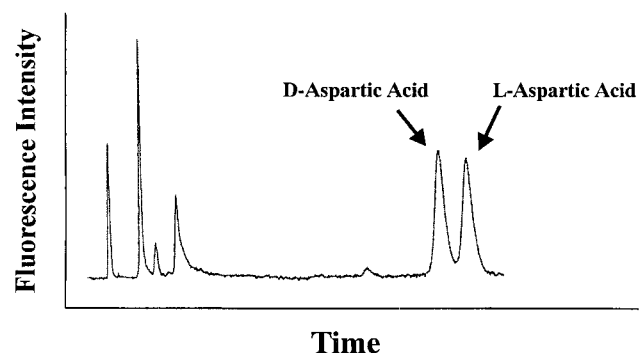


FIG. 1. Determination of the enantiomeric proportion of D-aspartic acid in the hyperthermophilic archaeum *Desulfurococcus* sp. strain SY. Aspartic acid purified from crude extract of *Desulfurococcus* sp. strain SY was subjected to enantiomeric separation by HPLC with a Pirkle-type chiral column. The experimental details are described in the text.

the proportion of D-amino acid (expressed as the ratio of D-isomers to total D- and L-isomers) was determined.

Significant amounts of D-aspartic acid in the crude extract of *Desulfurococcus* sp. strain SY were detected; the results are shown in Fig. 1. Aspartic acid was also highly racemized in *Thermococcus* sp. strains KS-1 and KS-8 and *Pyrococcus* sp. strains GB-D and OII: their D-aspartic acid contents were estimated to be 43.0, 48.4, 45.2 and 49.1%, respectively (Table 1).

Then, we determined the D-isomers of other amino acids in these hyperthermophilic archaea. Unexpectedly, we also detected D-enantiomers of amino acids such as Ala, Leu, Thr, Lys, and Phe in *Desulfurococcus* sp. strain SY and *Thermococcus* strains (Table 1). The percentage of D-isomers of alanine in *Thermococcus* strain KS-8 and leucine in *Thermococcus* strain KS-1 exceeded 20%. However, D-glutamic acid could not be detected in *Desulfurococcus* sp. strain SY or *Thermococcus* sp. strain KS-8.

Aspartate racemases are widely distributed among hyperthermophilic archaea. The accumulation of D-aspartic acid in the hyperthermophilic archaea suggested the existence of aspartate racemases. Thus, we examined aspartate racemase activity in these strains. Amino acid racemase activity was determined by measuring the D-amino acid produced from the L-amino acid. Reactions were performed at 70°C for up to 60 min in a 200- μ l reaction mixture containing 100 mM sodium-phosphate buffer (pH 8.0), 10 mM L-amino acid, and crude

TABLE 2. Aspartate racemase activity in hyperthermophilic archaea^a

Strain	Sp. act. (nmol/min/ mg of protein)
<i>Desulfurococcus</i> sp. SY	4.8
<i>Thermococcus</i> sp. KS-1	3.8
<i>Thermococcus</i> sp. KS-8	4.4
<i>Thermococcus</i> sp. KI	3.2
<i>Pyrococcus</i> sp. GB-D	5.5
<i>Pyrococcus</i> sp. OII	5.0

^a The specific aspartate racemase activity of the crude extract from hyperthermophilic archaea was measured at 70°C. The experimental details are described in the text.

extract of hyperthermophilic archaea. Reactions were started by the addition of crude extract and stopped by the addition of 800 μ l of methanol. The precipitated protein was removed by centrifugation at 4,500 \times g for 10 min. The amount of D-amino acid produced was determined as described above. Table 2 shows the aspartate racemase activity of hyperthermophilic archaea at 70°C. Activity was detected in all strains tested. The specific activity was in the range of 3.2 to 5.5 nmol/min/mg of protein.

Aspartate racemase genes of the hyperthermophiles. We attempted to find aspartate racemase genes of the hyperthermophilic archaea by PCR. Oligonucleotide primers were designed based on the consensus sequences between aspartate racemases of *Desulfurococcus* sp. strain SY and the lactic bacterium *Streptococcus thermophilus*. The forward primer [5' AT(ACT)-(CT)TN-GGN-GGN-ATG-GG 3'] is based on the amino acid sequence (Ile-Leu-Gly-Gly-Met-Gly), which is conserved in the N-terminal region of both aspartate racemases. Aspartate racemase is known to be cofactor independent and to use thiol groups of cysteine residues as bases (25). Two cysteine residues are conserved in aspartate racemases. However, one of them (Cys197 of *S. thermophilus*) has been proven not to be essential (25). Thus, the reverse primer [5' AA-(AG) (AT)A-(AG)TG-NGC-NGT-(AG)TT-(AG)CA 3'] is synthesized on the amino acid sequence [Cys-Asn-Thr-Ala-His-Phe-(Tyr)-Phe] around the essential cysteine (Cys84 of *S. thermophilus* and *Desulfurococcus* sp. strain SY). Using the primer set, we amplified DNA fragments of the predicted length from *Thermococcus* sp. strains KS-1 and KI and *Pyrococcus* sp. strain GB-D. The amplified fragment was subcloned into the pT7 Blue-T vector (Novagen) and sequenced. The amplified frag-

TABLE 1. Amino acid contents and D-amino acid proportions in hyperthermophilic archaea^a

Strain	Concn of amino acid (D-amino acid proportion)					
	Asp	Ala	Leu	Phe	Lys	Glu
<i>Thermococcus</i> sp.						
KS-1	26.03 (43.0)	17.79 (6.9)	1.03 (28.4)	1.22 (3.2)	7.95 (4.2)	NT
KS-8	18.61 (48.4)	8.47 (25.2)	0.89 (6.3)	0.15 (3.7)	17.24 (15.6)	ND (0.0)
<i>Pyrococcus</i> sp.						
OII	4.28 (45.2)	NT	NT	NT	NT	NT
GB-D	16.18 (49.1)	NT	NT	NT	NT	NT
<i>Desulfurococcus</i> sp.						
SY	8.99 (48.8)	4.54 (3.2)	0.89 (9.6)	0.15 (3.8)	2.84 (5.9)	ND (0.0)

^a Concentrations of free amino acids in crude extract are shown (values are in nanomoles/milligrams of protein). The proportion of D-isomer amino acids (expressed as the ratio of D-forms to total D- plus L-isomers) is shown in parentheses. The concentration of free glutamic acid for *Thermococcus* sp. strain KS-8 and *Desulfurococcus* sp. strain SY was not determined (ND). The experimental details are described in the text. NT, not tested.

ments were highly homologous to the aspartate racemase gene of *Desulfurococcus* sp. strain SY (data not shown).

Cloning and sequencing of the aspartate racemase gene from *Thermococcus* sp. strain KS-8. Then, we cloned the full-length gene of aspartate racemase from *Thermococcus* sp. strain KS-8. A clone, pKS8D301, with a *Pst*I/*Bam*HI fragment of about 4 kbp in pHSG298 (21), was obtained. It contained an open reading frame encoding a 232-residue polypeptide with a molecular weight of 25427.7. It shared considerable homology with the aspartate racemase of *S. thermophilus* (26) and was highly homologous to the aspartate racemase of *Desulfurococcus* sp. strain SY. Two cysteine residues and the surrounding amino acid sequences were highly conserved among these enzymes (data not shown).

To confirm that the cloned gene really encoded the aspartate racemase, the gene was expressed in *E. coli* by the T7 polymerase expression system (20). An oligonucleotide primer (5' CAT-ATG-CCG-GAG-CGC-GTC-ATC-GG 3') was designed to generate an *Nde*I digestion site at the initiation codon. Another primer (5' GGA-TCC-TTA-GAA-GTC-CTC-TAC-ACC-AA 3'), corresponding to the C-terminal end with a *Bam*HI digestion site, was synthesized. Using the primer set, a DNA fragment was amplified by PCR from the plasmid pKS8D301 and was subcloned into the pT7 Blue-T vector. The *Nde*I/*Bam*HI fragment of the constructed plasmid, pKS8D416, was excised and subcloned into pET23a to construct plasmid pKS8D514. The crude extract of the transformant containing pKS8D514 exhibited aspartate racemase activity at 70°C (data not shown).

A large portion of the aspartic acid in cells was converted to the D-enantiomer in *Thermococcus* and *Pyrococcus* strains. These strains have genes coding aspartate racemase and exhibit aspartate racemase activity. Recently, the total genomic sequence of *P. horikoshii* has been revealed (15, 16), and two genes homologous to the aspartate racemase gene of *Desulfurococcus* sp. strain SY were identified (data not shown). One of them (PH0670) is highly homologous to the aspartate racemase genes of *Thermococcus* sp. strain KS-8. Although *Desulfurococcus* sp. strain SY was originally classified into the genus *Desulfurococcus*, the 16S sequence showed that it should be included in the genus *Thermococcus* (17a). These results suggest that aspartate racemases exist ubiquitously and function in *Thermococcus* and *Pyrococcus* strains.

The sequences around the catalytic cysteine residue are not conserved in the aspartate racemase homologue of *A. fulgidus* (AF1422) and the 226-amino-acid-long hypothetical aspartate racemase of *P. horikoshii* (PH1733). Thus, further study is necessary to examine whether they really encode aspartate racemases or not. Amino acid racemase homologues were not found in the total genomic sequences of the methanogens *Methanococcus jannaschii* (2) and *Methanobacterium thermoautotrophicum* (19).

We have also found other D-amino acids in *Thermococcus* strains, including *Desulfurococcus* sp. strain SY (Table 1). Racemization is superficially a simple reaction. It is accomplished by the removal of an alpha hydrogen bound to a chiral carbon of the substrate and the subsequent nonspecific return of a hydrogen to the carbon. Amino acids are racemized only slowly under ordinary conditions; the half-lives of aspartic acid and alanine in racemization at 25°C are 3,500 and 12,000 years, respectively, because of the high dissociation energy of the C^α-H bond (1). However, racemization is greatly accelerated at a high temperature of around 100°C. For example, 20% of L-aspartic acid changes to the D-isomer in 1 day at 106.5°C (4). Goodfriend and Meyer measured racemization rates of amino acids at high temperatures and determined kinetic constants

(5). Using their values, ratios of the spontaneous racemization of the amino acids alanine, isoleucine, proline, aspartic acid, methionine, glutamate, and phenylalanine during culture (at 90°C for 1 day) were estimated. Under these conditions, a small portion of L-amino acid is racemized to the D-isomer. Among them, aspartic acid is most easily racemized and 1% of L-aspartic acid changes to the D-isomer. The kinetic constants of spontaneous racemization should change with the conditions, such as pH. However, the ratio of D- to total amino acids observed in this study could not be explained by spontaneous racemization under the culture conditions.

The presence of peptidyl D-amino acids in several kinds of archaea has been reported (18). However, the D-amino acid content was very low. It is possible that they were produced by spontaneous racemization during cultivation. Further study is required to elucidate the distribution of D-amino acids and amino acid racemases in archaea.

Although the existence of D-amino acids and amino acid racemases in *Thermococcus* and *Pyrococcus* strains has been proven with this study, their function is still unknown. It is believed that murein does not exist in *Archaea*. However, only a few studies have been performed on the cell envelopes of archaea, including strains of *Thermococcus* and *Pyrococcus* spp. Therefore, it is possible that some archaea have cell wall structures similar to those of bacteria and contain D-amino acids.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB015880 (*Thermococcus* sp. strain KS-8), AB022668 (*Thermococcus* sp. strain KS-1), AB022669 (*Thermococcus* sp. strain KI), and AB022670 (*Pyrococcus* sp. strain GB-D).

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