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Unusual enzyme characteristics of aspartyl-tRNA synthetase from hyperthermophilic archaeon *Pyrococcus* sp. KOD1

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Abstract **The *aspA* gene, encoding the aspartyl-tRNA synthetase (AspRS) from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1, was expressed in *Escherichia coli*. The KOD1 AspRS, which was purified to homogeneity and was shown to be functional in dimeric form, aminoacylated tRNA from KOD1. The optimum temperature for this activity was 65°C, which was lower than that for the cell growth of KOD1 (85°C). However, it increased to 75°C by the addition of polyamine molecules, such as putrescine, spermine, and spermidine. Analysis of the thermal denaturations of the enzyme and of KOD1-tRNA indicated that neither of them was denatured at temperatures below 70°C. These results suggest polyamine is one of the factors which are required to stabilize the AspRS-tRNA complex in vivo. In order to determine whether the nucleotide triphosphate (NTP) is required for Asp-tRNA synthesis, the aminoacylation was examined in the presence of each of the four NTPs. AspRS most effectively aminoacylated tRNA in the presence of ATP. However, we also found that the enzyme aminoacylated it even in the presence of GTP and UTP as well. Archaeon synthetase may have an interesting system to utilize other NTPs than ATP. The extreme conditions of early life may have given rise to these unique characteristics which then disappeared from developed organisms through evolution.**

Key words: Archaea; Hyperthermophile; Aminoacyl-tRNA synthetase; Evolution; Thermostability; *Pyrococcus*

1. Introduction

A study of a phylogenetic tree based on rRNA or protein sequences shows that all known organisms are related suggesting a common ancestor [1,2]. All organisms can be divided into three groups; eukarya, bacteria, and archaea. Phylogenetic location also suggests that the origin of life is thermophilic and also implies that hyperthermophilic microorganisms retain some physiological or biochemical features of early life forms. Studying characteristics of hyperthermophilic archaea enzymes must provide us with important information on an ancestral catalytic mechanism of life.

It is known that aminoacyl-tRNA synthetases (aaRS, EC 6.1.1), one of the key enzymes involved in the translation machinery, have significant homology within core motifs, and a comparison of amino acid (aa) sequences of these motifs reveals a divergent pattern between bacteria and eukarya [3–9]. These ubiquitous enzymes, which are indispensable in protein synthesis, catalyze two consecutive reactions: activation of carboxylic groups from natural aa, and the transfer of activated aa residues to the cognate transfer RNA (tRNA) molecules, leading to the formation of specific aminoacyl-tRNA [10].

Among aaRSs, aspartyl-tRNA synthetase (AspRS, EC 6.1.1.12) is one which has been well characterized in many organisms [11–13]. In order to understand the evolutional relationship of archaea, bacteria and eukarya, we cloned the AspRS-encoding gene from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1, which was recently isolated [14], and analyzed its sequence. Such data revealed that AspRS has a chimeric structure of eukaryotic and bacterial type enzymes [13]. It is suggested that its structure is an ancestral prototype before bacterial and eukaryotic enzymes were evolved. In this report, KOD1 AspRS was expressed in *E. coli*, purified and characterized for biochemical properties, including enzymatic activity.

2. Materials and methods

2.1. Overproduction and purification of recombinant KOD1 AspRS

The DNA fragment carrying the AspRS-encoding gene was amplified by PCR using two primers which have *Aff*III and *Bam*HI recognition sequences (5′-GTTGACATGTACAGGACGCACTACTA-3′ and 5′-CCAGGATCCGCAGGTTTATGTTGCTGGC-3′). The amplified DNA was cloned between *Nco*I and *Bam*HI sites of the plasmid pET-8c (Novagen, Madison, WI, USA) and the constructed plasmid was designated pLA556. The AspRS was overproduced in *E. coli* BL21(DE3) and purification was carried out based on the procedure of Poterszman et al. [15] with slight modifications. *E. coli* cells harboring pLA556 were induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at mid-exponential phase and incubated for 6 h at 37°C. The cells (from 3 liters of culture) were centrifuged and the pellet was washed with TS buffer (30 mM Tris-HCl [pH 8.0], 30 mM NaCl). Cells were disrupted by sonication and supernatant fraction was recovered by centrifugation at 27000×*g* for 20 min at 4°C. The supernatant was heat-treated at 80°C for 15 min and centrifuged again at 27000×*g* for 15 min at 4°C. The obtained supernatant was brought to 75% ammonium sulfate saturation and kept at 4°C overnight. The precipitate was collected by centrifugation at 27000×*g* for 30 min, dissolved in 20 ml of TS buffer and dialyzed overnight against the same buffer. The dialysate was applied to anion-exchange column (Resource Q, Pharmacia, Uppsala, Sweden) and equilibrated in TS buffer, and KOD1-AspRS was eluted by the linear gradient of NaCl using FPLC system (Pharmacia, Uppsala, Sweden). AspRS was further purified by repeating the same anion-exchange chromatography. The peak fraction was chromatographed with a gel filtration column (Superose 6, Pharmacia, Uppsala, Sweden) equilibrated with TS buffer at a flow rate of 0.5 ml/min. The molecular weight of the purified AspRS was also determined by analytical gel filtration on Superose 6 column.

2.2. Preparation of KOD1 tRNA

A hyperthermophilic archaeon strain KOD1 was isolated from a solfatara at a wharf of Kodakara Island, Kagoshima, Japan [14].

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Frozen KOD1 cells (20 g) were suspended in 50 ml of water and disrupted by adding 70 ml of phenol saturated with water. Phenol extraction was repeated twice and aqueous phase was obtained by centrifugation (12000×*g*, 20 min). Nucleic acids were precipitated by adding 0.1 volume of 20% potassium acetate (pH 5.0) and two volumes of ethanol. After centrifugation (12000×*g*, 20 min), the pellet was dissolved in 50 ml of 50 mM Tris-HCl (pH 7.5) and was incubated for 30 min at 37°C in the presence of 2 μg/ml DNase I. After chloroform extraction, tRNA was fractionated by DEAE cellulose column chromatography [16,17].

2.3. Enzymatic assays

Enzyme activity was examined by aminoacylation reaction using KOD1 tRNA as a substrate. Reaction was carried out according to the procedure of Eriani et al. [6]. The standard reaction mixture contained in 0.1 ml: 100 mM Tris-HCl (pH 7.5), 10 mM KCl, 2 mM ATP, 5 mM $MgCl_2$, 100 μM [^{14}C]L-Asp (211 mCi/mmol) and 25 μg of tRNA extracted from KOD1. After preincubation at reaction temperature, the reaction was started by the addition of 40 μl of the enzyme solution and the sample was incubated at reaction temperature for 10 min. The reaction was stopped by adding TCA and the mixture was kept in an ice box for 30 min. The solution was filtrated by Whatman 3MM filter paper. The radioactivity remaining on the filter was measured by liquid scintillation counting. One unit of activity is defined as the amount of enzyme producing 1 nmol of Asp-tRNA in 1 min. In order to identify aspartyl-tRNA (Asp-tRNA) apart from other components in the reaction mixture, gel filtration, instead of TCA precipitation, was performed using Bio-Gel P-6 (Bio-Rad, CA, USA).

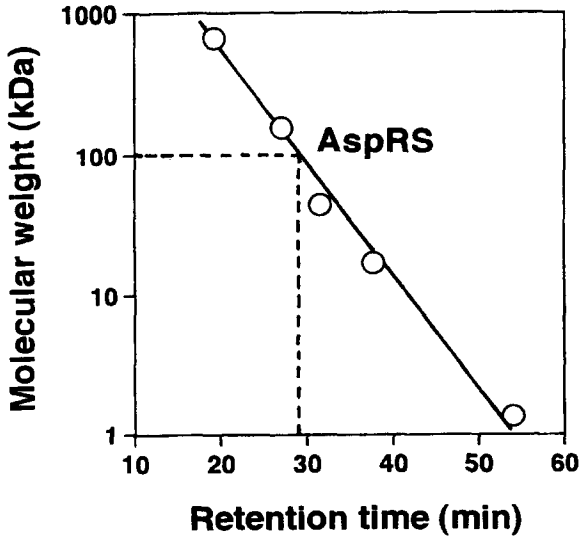

Fig. 2. Molecular weight determination by gel filtration. Thyroglobulin (670 kDa), gamma-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) were used as molecular weight standards.

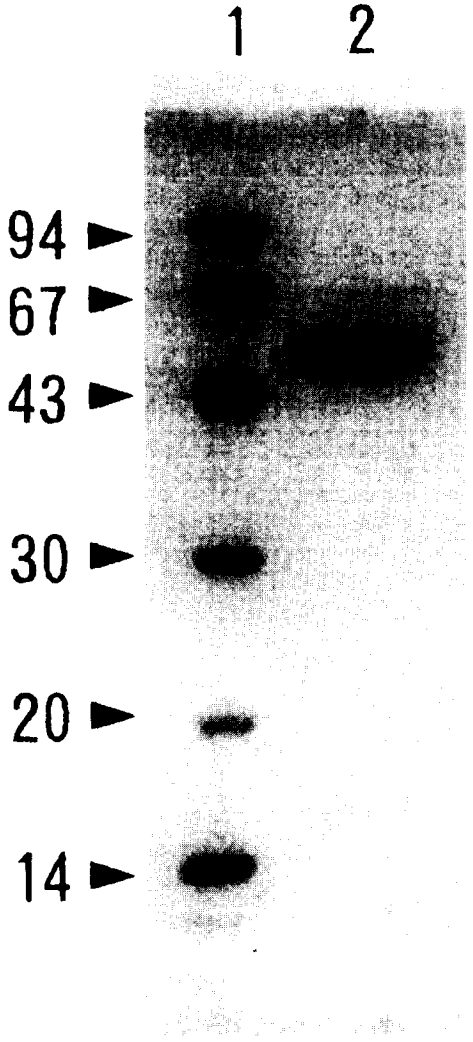

Fig. 1. The 0.1% SDS-12% PAGE of purified KOD1 AspRS. Lanes: 1, molecular weight markers; 2, purified AspRS. The gel was stained with Coomassie brilliant blue.

2.4. Circular dichroism

The circular dichroism (CD) spectra were measured on a J-720W automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.) based on the published procedure [18]. The far UV spectra (210–260 nm) were obtained using solutions containing proteins at 0.117 mg/ml in 10 mM Tris-HCl (pH 7.5) in a 2 mm optical path cell. The mean residue ellipticity, θ (measured in deg cm^2 $dmol^{-1}$), was calculated using an average amino acid molecular weight of 110.

2.5. Analysis of thermal denaturation

Thermal denaturation curves of KOD1-AspRS were determined by monitoring the change in the CD value at 220 nm using solution containing proteins at 0.117 mg/ml in 10 mM Tris-HCl (pH 7.5) in a 2 mm optical path cell as the temperature increased at a rate of 0.5°C/min. The temperature at midpoint of transition (*Tm*) was estimated from curve fitting on the resultant CD values versus temperature data.

Melting curves of KOD1 tRNA were measured in 10 mM sodium cacodylate buffer, pH 7.2, in the presence or absence of 20 mM $Mg(OAc)_2$ based on the method of Kowalak et al. [19]. In order to check an effect of divalent cations on tRNA thermostability, 14 mM CDTA was added in the absence of $Mg(OAc)_2$. The buffers were degassed by saturation with helium to prevent bubble formation. Optical melting transitions were measured with a spectrophotometer (U-3000, Hitachi Co. Ltd.) coupled to an NEC PC, capable of UV absorbance measurements at temperatures up to 100°C. The temperature was increased at a rate of 0.5°C/min and the absorbance was monitored at 258 nm.

3. Results and discussion

3.1. Enzyme characteristics

In order to elucidate the enzyme characteristics, the AspRS-encoding gene was expressed in *E. coli* and the enzyme was purified. The SDS-PAGE analysis was carried out as shown in Fig. 1, and the estimated molecular weight (50 kDa) agrees with the value deduced from sequence analysis (50893 Da) [13]. It was suggested that KOD1 AspRS possesses a Pro residue involved in dimer formation which is known to be a

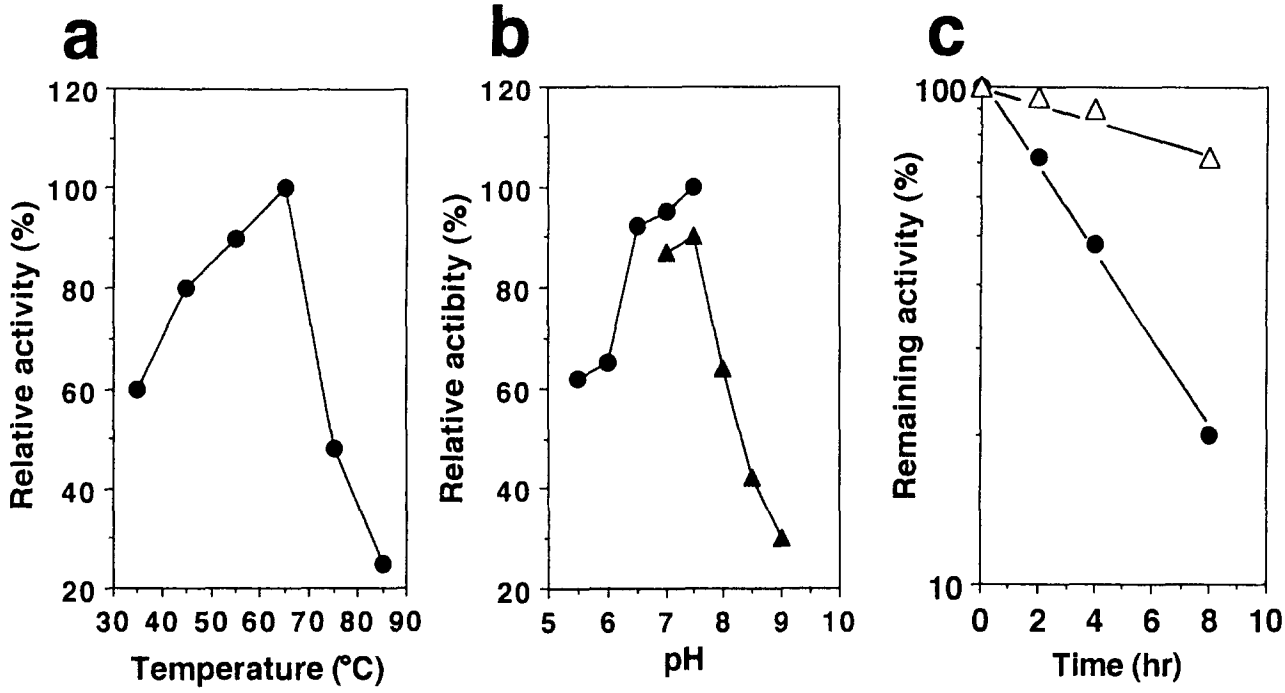

Fig. 3. Enzyme characteristics of purified KOD1 AspRS. (a) Effect of temperature on AspRS activity; (b) effects of pH on AspRS activity by 50 mM phosphate buffer (●) and 50 mM Tris HCl buffer (▲); (c) thermostability of KOD1 AspRS. The sample was incubated at 90°C (△) and 100°C (●) in 50 mM Tris HCl buffer (pH 8.0), and then the remaining activity was assayed at 65°C.

feature of all class-II enzymes [13]. Gel filtration pattern shows that the molecular weight of KOD1-AspRS is 100 kDa (Fig. 2), indicating that the enzyme acts as a dimer. The enzyme activity was examined by the aminoacylation reaction using tRNA from KOD1 as substrate. The optimum temperature and pH for the enzymatic activity were 65°C and 7.5, respectively (Fig. 3). The specific activity of purified AspRS was 110 U/mg at 65°C. The enzyme was very stable against heat inactivation. It had a half-life of 17 h at 90°C and 3.5 h even at boiling condition (100°C).

The optimum temperature for the enzymatic activity (65°C) is unexpectedly low, although optimum growth temperature of KOD1 is 85°C. In order to determine the thermostability of tRNA and AspRS from KOD1, thermal denaturation of tRNA and AspRS was examined by optical melting profile and circular dichroism, respectively. The conformational change of KOD1-tRNA was monitored by the absorbance at 258 nm as shown in Fig. 4. The thermal melting temperature (*Tm*) seems higher than 85°C in the presence of Mg^{2+}, however, in the absence of Mg^{2+}, melting temperature decreased to 85°C. Mg^{2+} is suggested to maintain the tRNA tertiary structure preventing melting of individual helices [20]. These results agree with those of *Pyrococcus furiosus* and *Pyrodictim occultum*. The *Tm* value of tRNA from *P. furiosus* is 96.9°C, which is 20°C higher than that predicted from its GC content. The tRNA fractionated from *P. furiosus* is known to have posttranscriptional modification of N^4-acetyl-2′-*O*-methylcytidine, N^2,N^2,2′-*O*-trimethylguanosine, and 5-methyl-2-thiouridine [19]. We suppose that the KOD1 tRNA also has a similar modification to prevent from thermal instability.

Thermal denaturation of KOD1-AspRS was examined by the CD spectra. The far UV CD spectra of the enzyme at 30°C and 90°C are shown in Fig. 5a. These results indicate that the conformation of the enzyme is changed when the temperature is increased from 30°C to 90°C. The thermal denaturation curve was then obtained by monitoring the change in the CD value at 220 nm. As shown in Fig. 5b, the CD value at 220 nm starts to increase at 70°C but does not reach a constant value even at 98°C. Therefore it seems

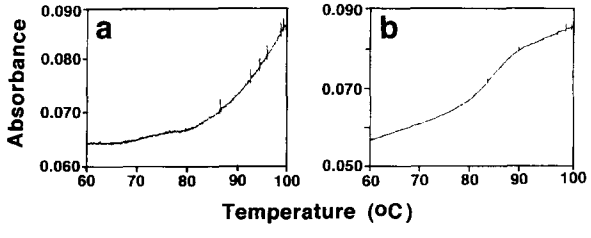

Fig. 4. Optical melting profiles of KOD1-tRNA in the presence (a) or absence (b) of Mg^{2+}. Absorbance was monitored at 258 nm.

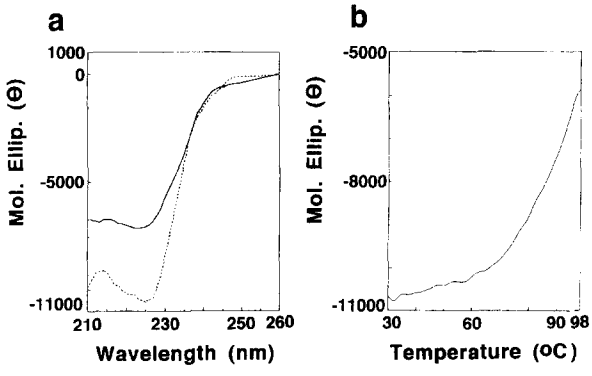

Fig. 5. CD spectra of KOD1-AspRS at various temperatures. (a) The far UV spectra at 30°C (dashed line) and 90°C (solid line). (b) Thermal denaturation curve at 220 nm.

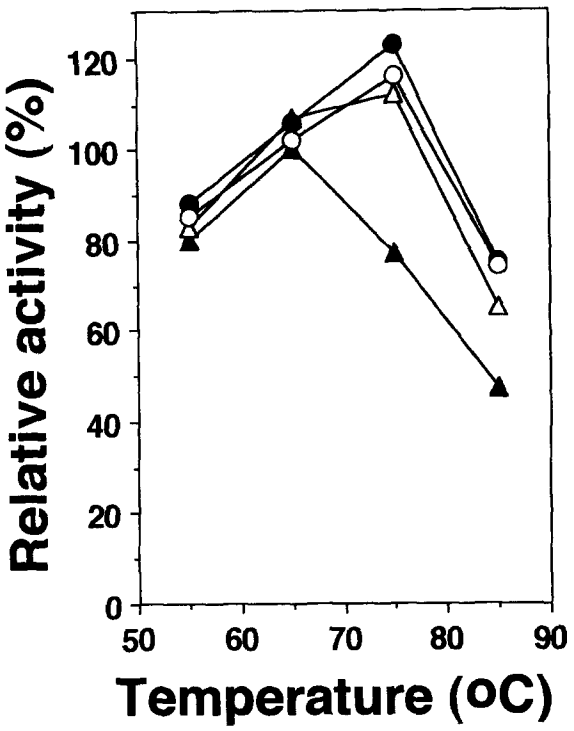

Fig. 6. Effect of polyamine on AspRS activity. AspRS assay was performed either without polyamine (▲) or with 70 μM putrescine (△), 5 μM spermine (○) and 70 μM spermidine (●).

clear that the enzyme starts to unfold at 70°C but is still not fully unfolded even at 98°C. Because the thermal unfolding of the enzyme is not fully reversible at this condition, we have not determined the thermodynamic parameters that characterize the thermal unfolding. However, the temperature of midpoint of transition (*Tm*) is apparently higher than the optimum temperature (65°C) for the enzymatic activity. Some unknown accessory may be involved in the AspRS-tRNA complex formation in the course of the enzyme reaction. Otherwise an active conformation of AspRS was maintained by the accessory at high temperature.

3.2. *Effect of polyamines*

Polyamines are known to be an important factor in maintaining the tRNA structure [21,22]. In some aminoacyl tRNA synthetase reactions, polyamines are required for their maximum activity [21]. Hyperthermophiles have relatively large amounts of polyamine, including unusual forms of polyamines which are found only in thermophiles [23–25]. In order to analyze the effect of polyamine molecules on the aminoacylation reaction, the reaction was carried out in the presence of putrescine, spermine, and spermidine at various concentrations. It was found that all polyamines enhanced the activity. The optimum concentrations of putrescine, spermine and spermidine were 70 μM, 5 μM, and 70 μM, respectively. In addition, the optimum temperature shifted from 65°C to 75°C in the presence of these polyamine molecules (Fig. 6). These results suggest that polyamines enhance enzymatic activity by stabilizing the conformation of the tRNA-AspRS complex. In hyperthermophiles, polyamines may play important roles in the AspRS reaction at extremely high temperatures.

3.3. *Requirement of NTPs*

Adenosine triphosphate (ATP) is known to be an energy source in various kinds of enzyme reactions. However, it is unknown why ATP was selected among various nucleotide triphosphate (NTP) molecules. Enzymes from hyperthermophilic archaea might possess features of an ancestral prototype in their reaction, suggesting that the reaction's energy source is not only ATP but also other NTPs. The aminoacylation reaction of tRNA proceeds through a two-step mechanism. The first step involves the formation of a stable aminoacyladenylate complex and subsequent release of pyrophosphate. In the second step, the 3′-terminal adenosine of enzyme-bound tRNA reacts with the aminoacyladenylate

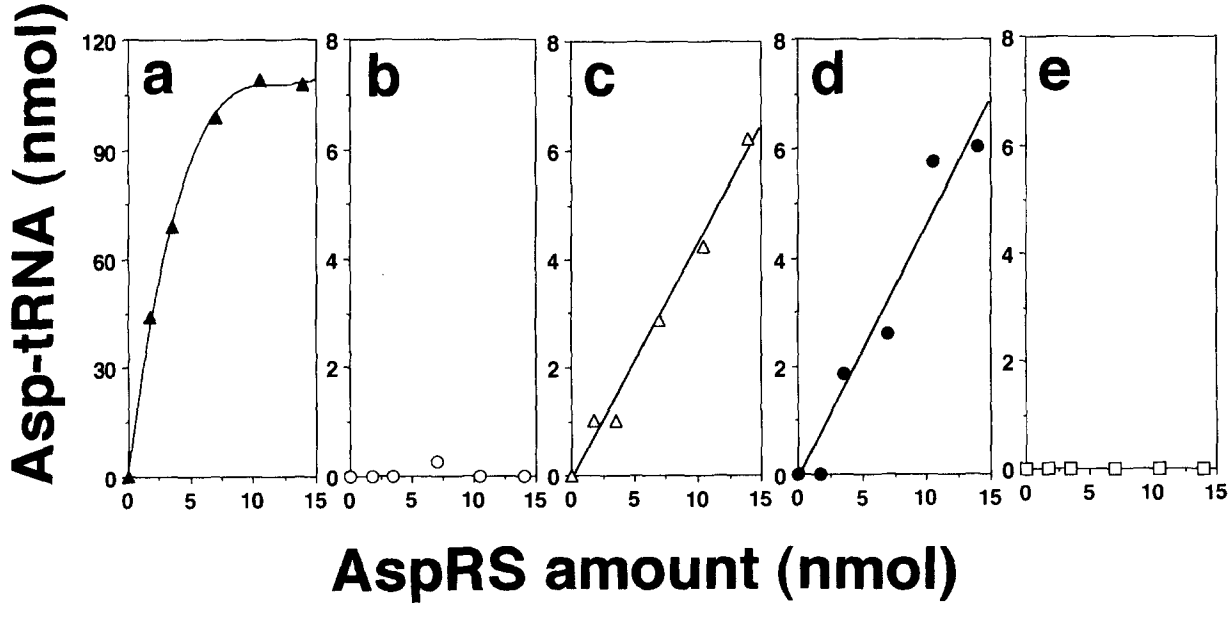

Fig. 7. Aspartyl-tRNA formation from NTPs at various amounts of AspRS. The amount of aspartyl-tRNA produced per min is indicated in the presence of ATP (a), CTP (b), GTP (c), and UTP (d) and in the absence of NTP (e).

to produce aminoacyl tRNA in the presence of a divalent cation, usually magnesium.

In order to determine the effect of NTPs other than ATP on aminoacylation, the aminoacylation reaction was carried out in the presence of GTP, CTP or UTP. Aspartyl-tRNA (Asp-tRNA), which was produced by the reaction, was separated by gel filtration and detected by liquid scintillation counting. When GTP and UTP were used, Asp-tRNA was produced, whereas when CTP was used, no Asp-tRNA was produced as shown in Fig. 7. These results suggest that KOD1-AspRS might form aminoacylguanylate and aminoacyluridylate as intermediates from the GTP and UTP, respectively. If so, this suggests that KOD1-AspRS might possess features of an ancestral prototype of today's evolved bacterial and eukaryotic enzymes. Broad NTP specificity of KOD1-AspRS makes us imagine that the synthetase of archaea can utilize NTPs beside ATP.

At present, the reason why KOD1-AspRS utilizes GTP and UTP besides ATP in an aminoacylation reaction is unclear. Possibly, ATP is easily denatured at high temperatures, at which hyperthermophiles grow, and the amount of available ATP might be limited. It may be reasonable that KOD1 possesses an ATP-independent system for the production of Asp-tRNA. In order to overcome the shortage in the amount of ATP at an extremely high temperature, KOD1-AspRS might possess flexibility to recognize and utilize other NTPs than ATP. Further studies are needed to prove such ideas.

It is known that KOD1-AspRS has a chimeric structure of bacterial and eukaryotic type AspRSs indicating that it may be an ancestral type enzyme [13]. From the perspective of evolution, the thermal environments, which are habituated by hyperthermophilic archaea such as KOD1, might have given rise to these initial organisms of life. These unique conditions would require organisms to have unusual characteristics, such as the high *Tm*, use of polyamines and consumption of various NTPs, in order to survive. These unusual characteristics would be lost through evolution as organisms were developed and proliferated in less extreme environments.

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References

[1] Woese, C.R. and Fox, G.E. (1977) Proc. Natl. Acad. Sci. USA 74, 5088–5099.
[2] Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Proc. Natl. Acad. Sci. USA 87, 4576–4579.
[3] Sellami, M., Fasiolo, F., Dirheimer, G., Ebel, J.-P. and Gangloff, J. (1986) Nucleic Acids Res. 14, 1657–1666.
[4] Gampel, G. and Tzagoloff, A. (1989) Proc. Natl. Acad. Sci. USA 86, 6023–6027.
[5] Mirande, M. and Waller, J.-P. (1989) J. Biol. Chem. 264, 842–847.
[6] Eriani, G., Prevost, G., Kern, D., Vincendon, P., Dirheimer, G. and Gangloff, J. (1991) Eur. J. Biochem. 200, 337–343.
[7] Moras, D. (1993) Biochimie 75, 651–657.
[8] Delarue, M., Poterszman, A., Mikonov, S., Garber, M., Moras, D. and Thierry, J.C. (1994) EMBO J. 13, 3219–3229.
[9] Kisselev, L.L. and Wolfson, A.D. (1994) In: Progress in Nucleic Acid Research and Molecular Biology (Cohen, W.E. and Moldave, K., Eds.) pp. 83–142, Academic Press, New York.
[10] Carter, C.W. (1993) Annu. Rev. Biochem. 62, 715–748.
[11] Cavarelli, J., Eriani, G., Rees, B., Ruff, M., Boeglin, M., Mitschler, A., Martin, F., Gangloff, J., Thierry, J.-C. and Moras, D. (1994) EMBO J. 13, 327–337.
[12] Poterszman, A., Delarue, M., Thierry J-C. and Moras, D. (1994) J. Mol. Biol. 244, 158–167.
[13] Imanaka, T., Lee, S., Takagi, M. and Fujiwara, S. (1995) Gene 164, 153–156.
[14] Morikawa, M., Izawa, Y., Rashid, N., Hoaki, T. and Imanaka, T. (1994) Appl. Environ. Microbiol. 60, 4559–4566.
[15] Poterszman, A., Plateau, P., Moras, D., Blanquet, S., Mazauric, M.-H., Kreutzer, R. and Kern, D. (1993) FEBS Lett. 325 183–186.
[16] Goldstein, J. (1971) In: Methods in Molecular Biology (Last, J.A. and Laskin, A.I., Eds.), Vol. 1, pp. 235–265, Marcel Dekker, New York.
[17] Gupta, R. (1995) In: Archaea, A Laboratory Manual (Robb, F.T., Place, A.R., Sowers, K.R., Schreier, H.J., DasSarma, S. and Fleischmann, J., Eds.) Vol. 2, pp. 119–131, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Kanaya, S. and Itaya, M. (1992) J. Biol. Chem. 267, 10184–10192.
[19] Kowalak, J., Dalluge, J.J., McClosky, J.A. and Stetter, K.O. (1994) Biochemistry 33, 7869–7876.
[20] Stein, A. and Crothers, D.M. (1976) Biochemistry 15, 160–168.
[21] Santi, D.V. and Webster, R.W. (1975) J. Biol. Chem. 250, 3874–3877.
[22] Jakubowski, H. (1980) FEBS Lett. 109, 63–66.
[23] Oshima, T. (1979) J. Biol. Chem. 254, 8720–8722.
[24] Oshima, T. (1982) J. Biol. Chem. 257, 9913–9914.
[25] Uzawa, T., Yamaguchi, A., Nishikawa, K. and Oshima, T. (1994) J. Biochem. 115, 830–832.