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Characterization of Recombinant Glutamine Synthetase from the Hyperthermophilic Archaeon *Pyrococcus* sp. Strain KOD1

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The *glnA* gene encoding glutamine synthetase was cloned from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1, and its nucleotide sequence was determined. The *glnA* gene was expressed in *Escherichia coli* ME8459 (*glnA* mutant strain), and the protein was purified to homogeneity and shown to be functional in a dodecameric form (637,000 Da), exhibiting both transferase and synthetase activities. However, kinetic studies indicated that the enzyme possessed low biosynthetic activity, suggesting that the reaction was biased towards glutamate production. The optimum temperature for both activities was 60°C, which was lower than the optimal growth temperature of KOD1. Recombinant KOD1 GlnA exhibited different optimum pHs depending on the reaction employed (pH 7.8 for the synthetase reaction and pH 7.2 for the transferase reaction). Of the various nucleoside triphosphates tested, GTP as well as ATP was involved in the synthetase reaction.

A study of a phylogenic tree based on rRNA or protein sequences shows that all known organisms are related, suggesting a common ancestor (42). All organisms can be divided into three groups: eukarya, bacteria, and archaea (43). Phylogenic locations also suggest that the origin of life is thermophilic and also imply that hyperthermophilic microorganisms retain some of the physiological or biochemical features of early life forms (12, 14, 29, 43). Studying characteristics of enzymes of hyperthermophilic archaea will provide us with important information for understanding ancestral catalytic mechanisms of life.

Glutamine synthetase (GS; EC 6.3.1.2) is known to be an important enzyme in living organisms. This enzyme plays a dual role by providing glutamine for biosynthesis and by assimilating ammonia. In most bacteria, glutamate dehydrogenase (GDH; EC 1.4.1.3), glutamate synthase (GOGAT; EC 1.4.1.13), and GS are used for ammonia assimilation. The catalytic reaction of each of these enzymes is as follows: for GDH, NH_3 plus 2-oxoglutarate plus NAD(P)H yields glutamate plus $NAD(P)^+$; for GS, NH_3 plus glutamate plus ATP yields glutamine plus ADP plus P_i; and for GOGAT, glutamine plus 2-oxoglutarate plus NAD(P)H yields 2-glutamate plus $NAD(P)^+$.

Under nitrogen-sufficient conditions, GDH mainly catalyzes glutamate production from 2-oxoglutarate and ammonia. However, under nitrogen-starvation conditions, GS produces glutamine from glutamate and ammonia and the produced glutamine molecules are then converted to glutamate by GOGAT. The GS-GOGAT pathway is the major route for utilization of ammonia when ammonia is deficient. GS has been extensively studied at both biochemical and molecular levels in various bacteria (2, 3, 9, 15, 16, 27, 37, 38), cyanobacteria (10), plants (8), and mammals (23). Although GSs from various organisms exhibit structural similarities, they differ in their mode of regulation. GS from members of the family *Enterobacteriaceae*, such as *Escherichia coli*, is subjected to posttranslational modification by adenylylation, and as the enzyme is progressively adenylylated, it becomes increasingly sensitive to feedback inhibition by metabolites downstream from glutamine (1, 44). In contrast, GSs from *Bacillus subtilis* (9), *Clostridium acetobutylicum* (18), and the thermophilic bacterium *Thermotoga maritima* (31) are not regulated by an adenylylation-deadenylylation mechanism.

GS characteristics of various organisms have been well studied. However, no report on the characterization of GSs from hyperthermophilic archaea is currently available. Only sequence information from hyperthermophilic archaea such as *Pyrococcus woesei* (40), *Pyrococcus furiosus* (5), and *Sulfolobus solfataricus* (30) is available. A recent report shows that *Thermococcus profundus* (20), which belongs to euryarchaeota, does not exhibit any detectable GS activity. This observation led us to investigate whether or not hyperthermophilic archaea use GS in an ammonia assimilation pathway. The enzyme may play a role only in providing glutamine for biosynthesis. In order to obtain a better understanding of the ammonia assimilation mechanism of hyperthermophilic archaea, we attempted enzyme analysis of recombinant GS from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1, which was isolated from a wharf on Kodakara Island, Kagoshima, Japan (24).

For GS gene cloning, PCR was performed with two degenerate oligonucleotide primers designed on the basis of conserved regions I and III. Sequencing analysis revealed the presence of an incomplete open reading frame encoding a protein which is highly homologous to other known *Pyrococcus* sp. GS genes. The remaining portion of the GS gene was obtained by gene walking. A 3.8-kbp *Bam*HI fragment containing the remaining part of the *glnA* gene was identified by Southern hybridization with a PCR-amplified fragment as a probe, and the nucleotide sequence was determined by the dideoxy chain termination method (32). The identified open reading frame was designated *glnA*. It was shown that *glnA* encoded a 443-amino-acid polypeptide, and it was classified into the GSI group (5, 22, 40). GlnA has a carboxyl-terminal extension which typically distinguishes GSI from GSII (1, 17). Five conserved regions, which are found in all GSs from both

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P.sp KOD1           M------NEIKGIERAVQVE--VPRPRFLLLAFTDINGSLKGMEIPM--------ER--Y
P.furiosus          M------N----ISVSMNKF--DSKIKFVQLVFVDINGMPKGMEIPA--------SR--L
P.woesei            M------N----ISVSMNKF--DSKIKFVQLVFVDINGMPKGMEIPA--------SR--L
S.solfataricus      MP-----S----TAEDVLKFLKENNIKWVDLQFTDVPGRLHHITIPA--------DEFDL
M.jannaschii        M-HWGMEM----NVEQAIEYVKKNNVKFIRFQFVDILGFPKNVAYPVK-AGEKGIEE--L
M.voltae            M------T----SLEMALEYIKINNVKFLRFQFVDIHGEPKNIAYPVKLGTADGEEE--L
                    *                               * *  *       *

P.sp KOD1           EEAVEDGVSFDGSSIPGFEGIEDSDLIFKADPSTYAEIPWE-G---IGRVY--GYIYKGD
P.furiosus          EEAVTDGISFDGSSVPGFQGIEDSDLVFKADPDTYVEVPWD-N---VARVY--GFIYKDN
P.woesei            EEAVTDGISFDGSSVPGFQGIEDSDLVFKADPDTYVEVPWD-N---VARVY--GFIYKDN
S.solfataricus      ESLKTGFGKLDGSSIRGFTSIYESDMVLLPVPETMTLIPWSPG---VARVLCKVFWGGGK
M.jannaschii        REIFENGVWFDGSSITGFVGIEESDMLLKPDLSTLSVLPWRPEEKSVARVICDVY-KDEK
M.voltae            MGVLENGLFFDGSSIEGFVEIEDSDMVLKPDLSTLSVLPWRPSEKSVARIICDVY-RKNG
                              ****  **  *  **        *    **        *
                           _______________________
                                      I
P.sp KOD1           EPYQADPRGILKRVLERLEKEGLKAHI-GPEPEFYIFK---K-----------------
P.furiosus          KPYGADPRGILKRALEELEKEGYKAYI-GPEPEFYLFK---K-----------------
P.woesei            KPYGADPRGILKRALEELEKEGYKAYI-GPEPEFYLFK---K-----------------
S.solfataricus      GRFERDPRFIAEEAEKYQTEQGYTSYY-GPELEFFMFD---KVKLDVSAPQSGTGYKIYA
M.jannaschii        TPFEGDPRSRLKAILEELKKEMNGEYFVGPEPEFFLLKRDPH-----------------
M.voltae            KPFEGDPRGCLKRVLAEFKEEFKGEYFVGPEPEFFILK---N-----------------
                         ***                    *** **

P.sp KOD1           -----NGTWELHIPDSGGYFDLVGLDKAREIRREIALYMP-YLGLKPEVLHHEVGKA-QH
P.furiosus          -----NGTWELEIPDVGGYFDILTLDKARDIRREIAEYMP-SFGLIPEVLHHEVGKA-QH
P.woesei            -----NGTWELEIPDVGGYFDILTLDKARDIRREIAEYMP-SFGLIPEVLHHEVGKA-QH
S.solfataricus      REAPWTDSGTFVIRFKEGYYPAPPVDQLMDVRVEIVDTLVKYFGYTIEATHHEVATAGQG
M.jannaschii        -----NP-HRWVPADDGGYFDVEPLDDAPDIRRDIVLALE-NLGFHVEASHHEVAPG-QH
M.voltae            -----EN-GKWVPGDDAGYFELEPLDEGNDLRRNIVFALE-NLGFHVEASHHEVAPG-QH
                                     **      *     *  *        *   *  ****    *
                                                                      __________
                                                                            II
P.sp KOD1           EIDFRYDEALRTADNIVSFKHVVKAVAELHGYYATFMPKPIYGFPGNGMHLHISLW-KDG
P.furiosus          EIDFRYDEALKTADNIVSFKYITKAVAEMHGLYATFMPKPLFGFPGNGMHLHISLS-KDG
P.woesei            EIDFRYDEALKTADNIVSFGYITKAVAEMHGLYATFMPKPLFGFPGNGLHLHISLW-KDG
S.solfataricus      EIDFRFSTLVDTADKVQTLKYVAKNIAAKHGLVVTFMPKPIYGDNGTGMHTHLSLWTKDG
M.jannaschii        EVDFKFDNALKTADSVITFKMTIKNIAKKHGLKATFMPKPFFGMNGNGMHCHQSVW-FNG
M.voltae            EVDFKYDNAVKTADSVITFKTTIKTLAKQSGVLATFMPKPFFGMNGSGMHCNQSIW-LDG
                    * **       ***         *  *   *   ******  *  * * *   *     *
                    ____                               _____________________
                                                                 III
P.sp KOD1           ENVFIGE-----DGLSDTALHFIGGILKHAKALAALTNPTVNSYKRLVPGYEAPVYISWG
P.furiosus          ENVFMGE-----EGLSEIALHFIGGILKHAKALIAVTNPTVNSYKRLVPGYEAPVYISWG
P.woesei            ENVFMGE-----EGLSEIALHFIGGILKHAKALTAVTNPTVNSYKRLVPSYEAPVYISWG
S.solfataricus      KKNLMYDPNDEYAEISQFGRYVIGGLLTHARALSAIVSPTVNSYRRLIPGFEAPVYIAWS
M.jannaschii        EPSF-YDPEGPYNGLSETCLSYIAGILSHAKALVAITNPTVNSYKRLVPGYEAPVNIAWA
M.voltae            KPSF-YDENNA-HQLSDICLSYIGGILEHTKALVSVTNPTVNSYKRLVPGYEAPVNIAWA
                                   *      * * * *  **     ****** ** *  ****  * *

P.sp KOD1           YRNRSALIRVPAF---KGSGARIEYRCPDPSANPYLALAGILMVGLDGIKKKVEPDSYVE
P.furiosus          YRNRSALIRVPAF---WGKGARIEYRCPDPSANPYFAFAAVLKAGLDGIKHKIDPFAYVE
P.woesei            YRNRSALIRVPAF---WGKGARIEYRCPDPSANPYFAFAAVLKAGLDGIKHKIDPFSYVE
S.solfataricus      KGNRSAVIRVPAYYRGMEKAKRIEYRPPDPSTNPYLAFAALLMAALDGVNKKIDPGDPVD
M.jannaschii        NKNRSAIIRVPAA---RGKATRIEFRAPDPTCNPYLAFACMLAAGLDGIKKKMTAPEPVE
M.voltae            NSNRTSIIRVPAA---RGKGTRVEFRAPDPACNPYLAFTVMLAAGLDGVRRKLIAPEPVE
                      **   *****         * * * ***  *** *    *   ***   *       *
                      __________         ___________________
                          IV                        V
P.sp KOD1           TNVYEMDDAERERLGIDTLPGSLGEALEELKKDKTVREALGGANKNFIDYKEREWEEYIE
P.furiosus          ENVYEMSEEKRKELGIETLPGSLGEALEELEKDKVVKEALGDAYKNFINYKWKEWESYLE
P.woesei            ENVYEMSEEKRKELGLETLPGNLGEALGELEKDKVVKEALGDSYKNFINYKWKEWESYLE
S.solfataricus      ENIYHLTPEKRRQLGIKELPRSLNEALDELESDK---EFLKPVFNSSI------LDTYID
M.jannaschii        RNIFKMSEEEKKQLGIESVPANLAAALDELECDE----VLQKALGKHI------YENYME
M.voltae            KNIFAMSEAEKREANIDSVPSNLYDAIEELRQDK----VLKKALGDHI------FNKFIE
                     *                 *  *  *  **  *      *       *

P.sp KOD1           YLSSRDIPIDTKKVTEWELERYFYV
P.furiosus          YLEEKHMPKDTKKVTEWELERYFFL
P.woesei            YLEEKHIPPDTEKVTEWELERYFFL
S.solfataricus      LKRDEARSLQG-YPHPMELYYYLDS
M.jannaschii        IKRAEWDDFRT-AVTDWETGKYLIY
M.voltae            IKTKEYDAYRT-AVTDWEFNKYVRI
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FIG. 1. Alignment of the deduced amino acid sequences of various archaeon GSs. GS sequences were obtained from either SwissProt or GenBank with the DNASIS program (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). Alignments of amino acid sequences were performed with the program ODEN, developed by the National Institute of Genetics (Mishima, Japan). Underlined regions indicate the conserved regions I, II, III, IV, and V, and asterisks indicate conserved residues among all the sequences. For KOD1 *glnA* gene cloning, PCR was performed with two degenerate oligonucleotide primers designed on the basis of conserved regions I and III, which possess the sequences 5′ GTT CTA GAT GGG ATA TCC TTG ACT CCTC 3′ and 5′ GGG AAT TCG AAG AG(G/T) GG(C/T) TT(T/A) GGC AT(A/G) AAT GTA GC 3′. PCR was performed as follows: 1 cycle consisted of 3 min (denaturation) at 96°C, 2 min (annealing) at 45°C, and 2 min (extension) at 72°C, and this cycle was followed by 29 cycles of 1 min at 96°C (denaturation), 1 min at 45°C (annealing), and 1 min at 72°C (extension). Amplification with these primers and under these conditions resulted in a 560-bp fragment. The DNA fragment obtained was used as a probe for Southern hybridization to screen the DNA fragment harboring the entire *glnA* gene from the KOD1 chromosome. The DNA sequences of both strands were determined by the dideoxy chain termination method (32). *P.*sp KOD1, *Pyrococcus* sp. strain KOD1; *M. jannaschii*, *Methanococcus jannaschii*; *M. voltae*, *Methanococcus voltae.*

prokaryotes and eukaryotes (1, 17, 22), were identified in KOD1 GlnA as shown in Fig. 1. These sites are associated with enzymatic functions. Conserved region III is considered to be an ATP-binding site (41) and is identified in KOD1 GlnA between residues 226 and 247. An adenylyltransferase recognition site which was found in *E. coli* GS (28, 36) was not observed in KOD1 GlnA, suggesting that KOD1 GS is not regulated by the adenylylation-deadenylylation mechanism.

In order to determine whether GlnA is functional as a GS, expression of *glnA* was performed in *E. coli* cells. However, the *glnA* gene was not successfully cloned in expression plasmid pET. Hence, the KOD1 *glnA* gene was cloned into plasmid pBR322 under the Tet^r gene promoter with *E. coli* ME8459 (a *glnA* mutant strain) as a host. The constructed plasmid (pKOD20) could be introduced into *E. coli* ME8459 but not into the JM109 ($glnA^+$) strain. These results suggested that overexpression of KOD1 *glnA* might have a toxic-titration effect on wild-type *E. coli* cell growth.

Purification of recombinant KOD1 GlnA was performed by monitoring the transferase activity of GS according to a previously reported assay method (3, 34). As shown in Fig. 2a, purification was achieved by heat treatment and repeating ion-exchange chromatography.

A sodium dodecyl sulfate (SDS)-polyacrylamide gel was stained with Coomassie brilliant blue. The KOD1 DNA fragment containing the *glnA* gene was amplified by PCR with two primers (5′ TGT CTA GAT GAA AGA AGGA GAT ATC CAT GAA CGA AAT TAA GGG AAT TGA GAG GGC GGT ACA AG 3′ and 5′ AAA GAT CTG AGG GAG CCC ATC AAA CGT AGA AGT 3′). The amplified DNA was digested with *Bgl*II and introduced into the *Eco*RV and *Bam*HI sites of pBR322. The constructed plasmid was designated pKOD20. *E. coli* ME8459 cells carrying pKOD20 were cultured at 37°C for 16 h in NZYCM medium (pH 7.0) (39) supplemented with 50 μg of ampicillin per ml. The cells were harvested by centrifugation and suspended in 10 mM imidazole hydrochloride (pH 7.0)–50 mM $MgCl_2$–1 mM mercaptoethanol (buffer A). After ultrasonic disruption of the cells, crude cell extract was obtained by centrifugation (30 min at 8,000 × *g*). The supernatant was incubated at 70°C for 10 min and centrifuged (30 min at 12,000 × *g*) to obtain crude extract.

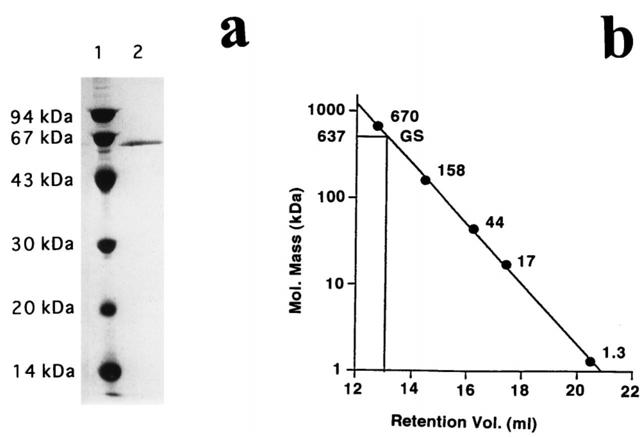

FIG. 2. Analysis by SDS-polyacrylamide gel electrophoresis and gel filtration chromatography. (a) Analysis by 0.1% SDS–12% polyacrylamide gel electrophoresis of the purified KOD1 GlnA. Lane 1, molecular mass standards, namely, rabbit muscle phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrate (30 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14 kDa); lane 2, purified KOD1 GS. (b) Molecular mass determination of the purified KOD1 GS. The molecular weight standards used were from Bio-Rad. The apparent molecular mass of the native enzyme was determined by analytical gel filtration on a Superose 6 column (model HR 10/30; Pharmacia Biochemical Inc.) equilibrated with 50 mM imidazole hydrochloride (pH 7.0)–50 mM NaCl–1 mM $MgCl_2$ at a flow rate of 0.2 ml/min. The column was calibrated with the following molecular weight standards: bovine thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000), and vitamin B_{12} (1,500).

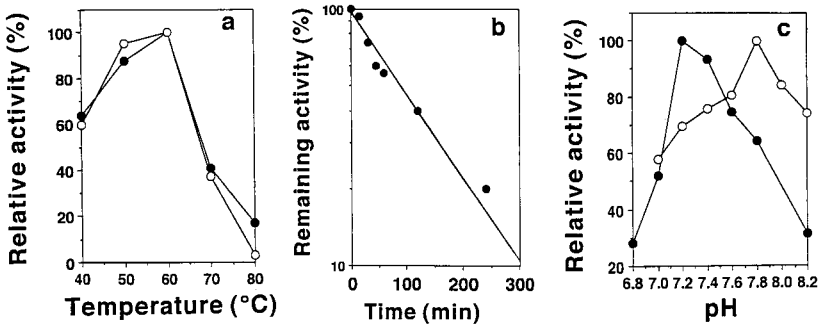

FIG. 3. Characterization of purified KOD1-GlnA. (a) Effect of temperature on synthetase (open circles) and transferase (filled circles) activities. In the synthetase activity assay, 50 μl of the enzyme was added to 0.4 ml of the reaction mixture containing 46 mM hydroxylamine hydrochloride, 60 mM $MgCl_2$, 164 mM monosodium L-glutamate, and 92 mM imidazole buffer (pH 7.0). The reaction was initiated with 60 μl of 0.2 M ATP (pH 7.0). As a control, ATP was replaced by water in one sample. The reaction was terminated by the addition of 1.0 ml of stop solution (55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, 21 ml of 11.6 N hydrochloric acid per liter). The precipitated protein was removed by centrifugation. One unit of GS activity was defined as the amount of enzyme producing 1 μmol of glutamylhydroxamate per min (1 μmol of glutamylhydroxamate gives an A_{540} of 0.533). In the γ-glutamyltransferase assay, enzyme solution (50 μl) or water was added to 0.4 ml of the assay mixture (18 mM hydroxylamine hydrochloride, 0.3 mM $MnCl_2$, 25 mM disodium hydrogen arsenate, 0.36 mM ADP, 135 mM imidazole hydrochloride). The mixture was then equilibrated for 5 min at the indicated temperatures, and the reaction was initiated by adding 50 μl of 0.2 M L-glutamine (final concentration, 20 mM). The reaction was terminated, and the absorbance was measured as described above for the synthetase assay. (b) Thermostability of GlnA at 60°C. The purified enzyme in Eppendorf tubes was placed in a water bath (60°C) for various periods and centrifuged, and the supernatant was obtained. The remaining activity (from the synthetase reaction) was assayed as described above. (c) Effect of pH on synthetase (open circles) and transferase (filled circles) activities. The influence of pH on both transferase and synthetase activities was determined in the presence of 135 and 92 mM imidazole hydrochloride buffer, respectively, over the pH range of 5 to 9.

The crude supernatant was brought to 80% saturation with solid ammonium sulfate and then stirred at 4°C for 3 h. The suspension was centrifuged (30 min at 12,000 × *g*), and the resulting pellet was suspended in 30 ml of buffer A. The supernatant was dialyzed overnight against the same buffer and centrifuged again to remove the precipitate formed during dialysis. The clear supernatant was applied to a Hi Trap Q fast protein liquid chromatography column (Pharmacia, Uppsala, Sweden) preequilibrated with buffer A. The enzyme was eluted with a linear gradient of KCl from 0 to 0.4 M KCl in buffer A at a flow rate of 1 ml/min. The active fractions were collected and dialyzed. Then the sample was loaded onto an anion-exchange column, POROS HQ (PerSeptive Biosystems, Cambridge, United Kingdom), equilibrated with buffer A, and eluted at a flow rate of 1 ml/min with a linear gradient of 0 to 0.4 M KCl in the same buffer. The active fraction was collected and dialyzed against buffer A and stored at 4°C.

In order to know the exact molecular mass of the purified protein in Fig. 2a, an analytical gel filtration column was used. GlnA was eluted as a single peak, with a retention volume showing a molecular mass of about 637,000 Da, which indicates that the enzyme is a homooligomeric enzyme composed of 12 molecules (Fig. 2b).

The effect of reaction temperatures on GS activity in both the synthetase and the transferase reaction is shown in Fig. 3a. The optimum temperature for both reactions was 60°C. The enzyme has a half-life of 98 min at 60°C, as shown in Fig. 3b. The optimum temperature for the activity of the enzyme (60°C) is rather low, compared with the optimum temperature for growth of KOD1 (95°C). In addition, KOD1 GlnA did not exhibit high thermostability in an in vitro experiment (Fig. 3b). Recombinant KOD1 GlnA synthesized in *E. coli* might not be fully folded. The lack of stability at high temperature may reflect incomplete folding. Most enzymes and proteins from hyperthermophiles seem to be thermostable; however, some intracellular enzymes are known to be unstable at an ambient temperature (19, 25). Hyperthermophiles possess cytoplasmic thermoprotectant solutes, such as high concentrations of K^+ ion and the novel sugar di-inositol-1,1′-phosphate (33) or tri-anionic cyclic 2,3-diphosphoglycerate (4, 13), that minimize denaturation. These carbon compounds might play a role in stabilizing GlnA against denaturation in vivo and in vitro.

The effect of various pHs on GS activity was examined by a synthetase and transferase assay system as shown in Fig. 3c. The pH optima are rather narrow, with the synthetase reaction (in which Mg^{2+} was the divalent cation) exhibiting a pH optimum of about 7.8 and with the transferase reaction (with Mn^{2+}) exhibiting a pH optimum of 7.2. The pH profile of enzyme activity shows different pH optima depending on the reaction employed. The exact mechanism responsible for this pH-dependent control remains to be investigated. However, this observation suggests that nitrogen assimilation through the glutamate-glutamine pathway might be controlled by the cytoplasmic pH difference.

ATP is known to be a widely used energy source in synthetase (ligase) reactions. It is unknown why ATP was selected over the various nucleoside triphosphate (NTP) molecules available. Moreover, it is thought that enzymes from hyperthermophiles might possess biochemical features of an ancestral prototype which lived under extreme limiting environments, suggesting that the reaction energy source is not only ATP but also other NTPs. Some enzymes from hyperthermophilic archaea are known to utilize NTPs or pyrophosphate besides ATP as the source of their reaction energy (11, 21). The ability of various NTPs to replace ATP in the synthetase reaction of KOD1 GlnA was examined. As shown in Table 1, GTP is about 78% effective in replacing ATP as an energy

TABLE 1. Effect of NTPs on GS activity measured after a synthetase reaction

NTP	Concn (mM)	Activity (μmol/min)	Relative activity (%)
None[a]	0	0	<1
ATP	30	0.460	100
GTP	30	0.360	78
CTP	30	0.003	<1
UTP	30	0.004	<1

[a] The reaction was carried out in the absence of NTP.

TABLE 2. Kinetic parameters of KOD1 GS in synthetase and transferase reactions[a]

Reaction type	Substrate	Concn range (mM)	K_m (mM)	k_{cat} (10^2 min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Synthetase	L-Glutamate	1–30	23.5	21.9	93.2
	Hydroxylamine	5–30	15.2	7.7	38.5
	Hydroxylamine	0.1–5	1.6	7.7	481.2
	ATP	1–100	28.0	31.6	112.9
Transferase	L-Glutamine	1–50	5.0	39.0	780.0
	ADP	0.1–10	6.3	7.2	114.3

[a] In the synthetase reaction, Mg^{2+} was used as the divalent cation, and in the transferase reaction, Mn^{2+} was used as the divalent cation. The kinetic constant for substrates was determined at 60°C and at pHs 7.2 and 7.8 for the transferase and synthetase reactions, respectively.

source for the binding of glutamate. Other NTPs tested showed no activity when assayed, indicating the enzyme's preference for purine residues over pyrimidines. *B. subtilis* GS can replace ATP with other NTPs depending on the divalent cation used (9). In reactions in which Mn^{2+} was used, GTP, UTP, and CTP had a limited ability to replace ATP (less than 30%), while in reactions in which Mg^{2+} was used, complete inhibition by these NTPs was observed. In *E. coli* GS, replacing ATP with GTP produced 30% activity in the synthetase reaction (44). KOD1 GlnA exhibited significant efficiency in replacing ATP with GTP (78%). Another enzyme from KOD1, aspartyl-tRNA synthetase, aminoacylated tRNA even in the presence of GTP and UTP (11). Broad NTP specificity of hyperthermophilic archaeon synthetases suggests that enzymes of ancestral life forms can utilize various NTPs besides ATP.

Most hyperthermophiles show a very high level of GDH activity in producing glutamate (6, 20, 26). GDH in KOD1 cells is also very abundant (about 11% of total soluble protein [data not shown]), suggesting that the major pathway for ammonia assimilation is through GDH. These findings also suggest that hyperthermophilic archaea do not utilize GS predominantly for ammonia assimilation. Table 2 shows the kinetic constants (K_ms) for KOD1 GlnA's GS with respect to the substrates glutamate, hydroxylamine, ATP, glutamine, and ADP as determined by synthetase and transferase reactions. The K_m value for glutamate is 23.5 mM, which is relatively higher than that for glutamine (5 mM), suggesting the enzyme's clear preference for glutamine rather than glutamate. Such properties were also found in GS kinetic properties of *Rhizobium meliloti* GSIII (35). In our work, the K_m value of GlnA for hydroxylamine was higher when a high concentration was used (5 to 30 mM). These kinetic values show that the reaction is biased towards the degradation of glutamine under ammonia-rich conditions. The enzyme may have a minor biosynthetic role in vivo. However, with a lower concentration of hydroxylamine (0.1 to 5 mM), the enzyme shows higher affinity for ammonia and higher catalytic activity in forming glutamine (the catalytic constant [k_{cat}] value for both concentration ranges is 7.7×10^2 per min). This result suggests that KOD1 GlnA might play some role in ammonia assimilation under ammonia-starvation conditions. Glutamate synthase (GOGAT) also seems to be involved in this pathway. The kinetic analysis of these enzymes (GOGAT and GDH) will provide more-detailed information on the ammonia assimilation pathway of hyperthermophilic archaea. GDHs of *P. furiosus* and *S. solfataricus* also show two K_m values corresponding to high and low concentrations of ammonia (6, 7). With *S. solfataricus* GDH, the existence of several conformational states has been considered (7). There are several possibilities to explain the two-phase affinity of KOD1 GS. Multiple forms of the enzyme which catalyze the same reaction at the same time with different kinetic parameters, like GDHs of hyperthermophilic archaea, may exist. The substrate and cofactor may not follow an ordered mechanism of binding and may release at a high concentration of substrate. Further study is needed to investigate such a hypothesis with KOD1 GS.

Nucleotide sequence accession number. Sequence data has been deposited in the EMBL/DDBJ/GenBank data library under accession no. D86222.

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