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The Adenylate Kinases from a Mesophilic and Three Thermophilic Methanogenic Members of the Archaea

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Adenylate kinase has been isolated from four related methanogenic members of the Archaea. For each, the optimum temperature for enzyme activity was similar to the temperature for optimal microbial growth and was approximately 30°C for *Methanococcus voltae*, 70°C for *Methanococcus thermolithotrophicus*, 80°C for *Methanococcus igneus*, and 80 to 90°C for *Methanococcus jannaschii*. The enzymes were sensitive to the adenylate kinase inhibitor P^1,P^5-di(adenosine-5′)pentaphosphate, a property that was exploited to purify the enzymes by CIBACRON Blue affinity chromatography. The enzymes had an estimated molecular mass (approximately 23 to 25 kDa) in the range common for adenylate kinases. Each of the enzymes had a region of amino acid sequence close to its N terminus that was similar to the canonical P-loop sequence reported for all adenylate kinases. However, the methanogen sequences lacked a lysine residue that has previously been found to be invariant in adenylate kinases, including an enzyme isolated from the archaeon *Sulfolobus acidocaldarius*. If verified as a nucleotide-binding domain, the methanogen sequence would represent a novel nucleotide-binding motif. There was no correlation between amino acid abundance and the optimal temperature for enzyme activity.

The methanogens are those members of the Archaea whose defining metabolic characteristic is the production of methane (1, 30). The biochemistry of methanogenesis is well understood, and the process is known to entail the participation of novel coenzymes and electron carriers in an electron transport chain that is unique to these obligate anaerobic microbes (9, 27). The mechanism by which these methanogens couple electron transport reactions to ATP production has been investigated in recent years, and for those organisms characterized, it is apparent that ATP formation is coupled to a chemiosmotic ion gradient generated through the translocation of protons and/or sodium ions through the activity of primary ion pumps energized by the exergonic reactions of methanogenesis (for a review, see reference 3). In the case of *Methanococcus voltae*, a focus of this study, it has been suggested that ATP production can be entirely sodium ion-based through the mediation of a primary sodium ion pump coupled to a sodium ion-translocating ATP synthase (11). Nevertheless, while an ATP synthase has been purified from this methanogen, it has not been established that this enzyme catalyzes sodium ion translocation (7).

Despite progress in the characterization of electron transport-driven ATP production in methanogens, little is known about additional aspects of metabolic energy storage, conservation, and transfer in methanogens. Nevertheless, adenylate kinase (EC 2.7.4.3) activity has been reported in both *Methanobacterium thermoautotrophicum* and the methanogenic strain Gö1 (8, 23), and so it is expected that this enzyme will be present in other methanogens, where it mediates the interconversion of adenine nucleotides according to the formula ATP + AMP↔ADP. To date, there is only a single report of a characterized adenylate kinase isolated from an Archaeon (16, 18). The source of that enzyme was *Sulfolobus acidocaldarius*, a thermoacidophile member of the Crenarchaeota branch of the Archaea (30) that grows optimally at 75 to 80°C and pH 2 to 3 (4). In this study, we report the purification and partial characterization of the adenylate kinase isolated from four phylogenetically related marine methanogenic members of the Euryarchaeota branch of the Archaea, the mesophile *M. voltae* and the thermophiles *M. thermolithotrophicus*, *M. igneus*, and *M. jannaschii* (5, 13, 15, 29).

MATERIALS AND METHODS

Archaeal strains and cultivation. *M. voltae*, *M. jannaschii*, *M. thermolithotrophicus*, and *M. igneus* were grown at 30, 82 to 85, 65, and 80°C, respectively, as described previously (5, 13, 15, 29). In all cases, the cells were grown anaerobically in an atmospheric gas phase of 20% CO_2 and 80% hydrogen as the substrate.

Purification of adenylate kinase. A 10% culture inoculum was transferred to fresh medium (usually 3 liters), and the culture was grown to the early stationary phase (approximately 10^9 cells per ml). Cells were harvested aerobically by centrifugation, washed with buffer A (50 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 50 mM KCl, 400 mM NaCl) and resuspended in buffer B (20 mM Tris-Cl [pH 8], 10% glycerol) to about 1/200 of the original culture volume. After addition of DNase and phenylmethylsulfonyl fluoride (Sigma; final concentration, 1 μg/ml each), the suspension was passed through a chilled French pressure cell at 41.2 MPa for *M. voltae* and at 55 MPa for the other methanogens. The cell lysate was next centrifuged at 10,000 × *g* for 15 min to pellet debris and unbroken cells, and the supernatant fraction was collected as the crude extract. The crude extract was separated into the crude membrane fraction and the cytosol by centrifugation at 346,000 × *g* for 30 min at 4°C in a Beckman TL-100 tabletop ultracentrifuge with a TLA-100.3 rotor.

Affinity chromatography was performed at room temperature on a CIBACRON Blue (Sigma) column (40 ml). In general, about 50 mg of cytosolic extract protein was applied to the column. After washing the protein in buffer B to remove unabsorbed material, we next eluted adsorbed protein by using a step NaCl gradient. The pooled fractions containing adenylate kinase activity (the 400 to 600 mM NaCl fraction) were desalted and then reapplied to a CIBACRON Blue column, and the adenylate kinase was eluted with buffer B containing P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap_5A; 100 to 300 μM, depending on the particular enzyme). Although this procedure was used in our early preparations, we have more recently eliminated the NaCl washing step and now elute the protein contained in the cell extract directly with Ap_5A. In the case of the *M. jannaschii* enzyme, we found that warming the enzyme to 60°C enhanced its adsorption to the CIBACRON Blue. The pooled sample from the previous step was concentrated and then applied (about 0.5 mg of protein) to a 1-ml FPLC-Mono Q column (HR 5/5) Pharmacia) that had been equilibrated with buffer B adjusted to pH 9.0. Elution was done by a 0 to 1 M NaCl linear gradient in buffer

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TABLE 1. Properties of the purified methanococcal adenylate kinases

Species	Growth temp (°C)	Sp act (μmol of ATP mg^{-1} min^{-1})	Temp range for max. activity (°C)	$t_{1/2}$[a] (min)	
				68°C	89°C
M. voltae	30	14	30–40	4	1.5
M. thermolithotrophicus	65	81	60–80	25	6.0
M. jannaschii	82–85	89	70–90	30	6.0
M. igneus	80	56	70–90	43	17.0

[a] $t_{1/2}$, time required for 50% inactivation.

B (pH 9.0). Adenylate kinase activity eluted at 230 to 360 mM NaCl, depending on the enzyme. The purified enzymes were next concentrated in a Centricon microconcentrator (10,000-molecular-weight cutoff; Amicon). The purified enzymes could be stored in buffer B at 4°C under aerobic conditions for at least 5 months without loss of activity.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (19). Protein was visualized by silver stain. The molecular weight of the purified adenylate kinases was estimated by extrapolation to a standard curve based on the migration distance of proteins of known size. The native gel was of identical ionic composition as the denaturing gel except that it lacked SDS and mercaptoethanol and the samples were not heated. Adenylate kinase ADK was assayed on native gels by the agar overlay method (6).

Amino acid sequencing and amino acid analysis. The protein to be analyzed was subjected to SDS-PAGE and then electroblotted to a polyvinylidene difluoride membrane with a Bio-Rad electroblotting apparatus. After transfer, the protein bands of interest were excised from the membrane and applied to a pulsed-liquid phase protein sequencer with on-line phenohydantoin analyzers (Applied Biosystems) for amino acid sequencing. Amino acid analysis was performed on a Hewlett-Packard Amino Quant 1090 analyzer. Both analyses were carried out in the Genetic Engineering Facility of the University of Illinois Biotechnology Center.

Assays. Adenylate kinase activity was determined in both the forward and backward directions by following either the formation or depletion of ATP in the reaction mixture by the luciferin-luciferase method with a Turner TD-20E luminometer (11). In all cases, the reaction was initiated by the addition of the substrate, and samples were withdrawn for ATP assay 15 s later by removal into 12% (wt/vol) perchloric acid. The reactions were linear for up to several minutes, and the specific activities as well as the temperature optima reported in Table 1 are based on the average of several 15-s time points. For the temperature inactivation studies, enzyme activity was assayed by determining the ATP-dependent formation of reduced NADPH in a coupled assay mixture that contained glucose, hexose kinase, glucose-6-phosphate dehydrogenase, and NAD^+ (24). Corrections were not made for the relative stability of ATP at the various temperatures of incubation, but in separate experiments, we determined that ATP stability did not differ significantly between 40 and 80°C under our assay conditions. Protein was measured by the method of Lowry et al. (21).

Temperature inactivation studies. Enzyme (1 to 15 μg of protein, depending on the enzyme preparation) in 0.25 to 0.5 ml of 50 mM Tris-HCl (pH 7.7 at 25°C)–100 mM KCl–2 mM $MgCl_2$ was incubated in polypropylene tubes with a screw-top (Sarstedt Inc., Newton, N.C.; catalog number 72.694.006). Each experiment was initiated by adding a 12.5- to 25-fold dilution of stock enzyme solution (maintained at 4°C) to incubation buffer that had been warmed to the assay temperature. After incubation for the desired time in a Temp-Blok Module Heater (Scientific Products), each sample was chilled in an ice-water bath. In all cases, the sample temperature was reduced to below 15°C in less than 0.5 min. After addition of ADP (2.5 mM, final concentration), the samples were incubated at 40°C (*M. voltae*), 68°C (*M. thermolithotrophicus*), or 83°C (*M. jannaschii* and *M. igneus*) for 20 min, and the amount of ATP formed was determined by the coupled assay described above. The control sample was enzyme incubated on ice for the full duration of the inactivation phase of the experiment. No correction has been made for loss of enzymatic activity that may have occurred during the activity assay phase of the experiments. The results in Table 1 represent averages of at least two independent experiments. In the two cases tested, *M. voltae* and *M. jannaschii*, similar results were obtained with independent preparations of pure enzyme.

RESULTS

Identification of adenylate kinase activity in *M. voltae*. We initially observed adenylate kinase activity in membranes prepared from French press-disrupted *M. voltae*. However, subsequent studies showed that more than 90% of the activity found in the crude cell extract was present in the cytosolic fraction prepared by removal of membranes by centrifugation at 300,000 × *g* for 30 min and that further washing of the membranes with buffer (10 mM Tris [pH 8], 10 mM $MgCl_2$, 80 mM KCl, 100 mM NaCl, 200 mM sucrose) resulted in a membrane fraction that was free of detectable enzyme activity.

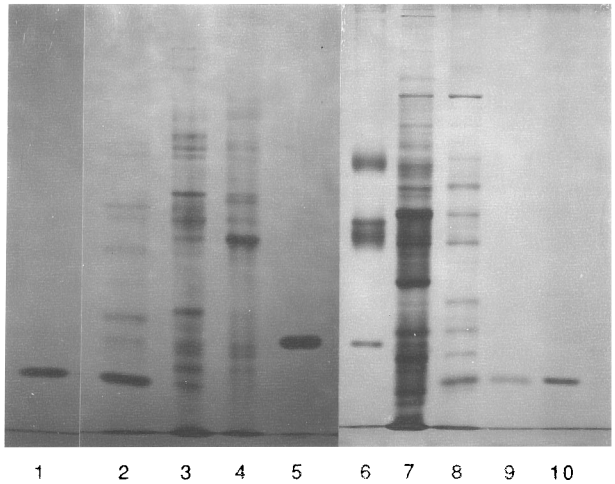

FIG. 1. SDS-PAGE. Lanes 1 to 4, *M. voltae*. Lane 1, purified enzyme after MonoQ chromatography (3 μg); lane 2, pooled fractions (10 μg) from CIBACRON Blue affinity chromatography eluted with Ap_5A; lane 3, pooled fractions (16 μg) from CIBACRON Blue affinity chromatography eluted in the 400 to 600 mM NaCl fraction; lane 4, crude extract (25 μg); lane 5, Sigma molecular weight marker (10 μg, carbonic anhydrase, 29 kDa); lane 6, Sigma molecular weight markers (10 μg each of carbonic anhydrase, 29 kDa; egg albumin, 45 kDa [heterogeneous because of glycosylation]; and bovine albumin, 66 kDa); lanes 7 to 9, *M. jannaschii*. Lane 7, crude extract (15 μg); lane 8, pooled fractions (7.5 μg) from CIBACRON Blue affinity chromatography eluted with Ap_5A; lane 9, purified enzyme (2 μg) after MonoQ chromatography. Lane 10, Purified *M. voltae* enzyme (2 μg). Proteins were visualized by silver staining.

Purification and properties of the *M. voltae* adenylate kinase. The enzyme was purified by a combination of CIBACRON Blue affinity and FPLC-Mono Q chromatography. In the first step, membrane-free cell extract was applied to a CIBACRON Blue column, and the unabsorbed proteins were eluted with buffer of low ionic strength (see Materials and Methods). Subsequent elution by buffer containing Ap_5A, a specific inhibitor of adenylate kinase (20, 22), led to elution of adenylate kinase activity together with other contaminating proteins. This step led to an approximately 100-fold increase in specific activity. In the second step, pooled enzyme activity from the first step was applied to an FPLC-Mono Q column, and the adenylate kinase was eluted with a salt gradient (elution at 230 mM NaCl). These procedures yielded an enzyme preparation that had a specific activity of approximately 10 μmol of ATP min^{-1} mg^{-1} (Table 1). The purified enzyme had a specific activity that was 135 times that found in the cell extract, and the total yield was 11.5%. The purified enzyme migrated in SDS-PAGE analysis as a single band of approximately 25 kDa whether visualized by silver staining or Coomassie brilliant blue. A comparison of the proteins present at each stage of the purification is shown in Fig. 1. We also observed that the purified protein migrated as a single protein band on a native PAGE system and that the band migrated to a position that was identical to that found for adenylate kinase activity found in the cell extracts as assayed by activity staining.

While the initial enzyme assays were carried out under strictly anaerobic conditions in an atmosphere of either 80% H_2–20% CO_2 or 100% N_2, the enzyme was fully active in air.

Activity was Mg^{2+} dependent, and the addition of Ap_5A inhibited enzymatic activity (50% inhibition at 50 μM Ap_5A) whether assayed in the direction of ATP formation from ADP or of ATP conversion to ADP. As expected for an adenylate kinase, ADP-dependent ATP production was totally inhibited by the addition of 1 mM AMP (data not shown). The enzyme showed similar activity over a pH range from 6 to 10, which was not a surprising result because it is known that the internal pH of *M. voltae* can vary from 6.5 to 8.5 depending upon the pH at which the strain is grown (11).

Purification of adenylate kinases from other methanogens. Since we wished to compare the adenylate kinases of mesophilic and thermophilic methanococci, we used the procedure described above to purify the enzyme from *M. thermolithotrophicus*, *M. igneus*, and *M. jannaschii*, whose optimal growth temperatures are approximately 65, 85, and 88°C, respectively. For each methanogen, Ap_5A-sensitive adenylate kinase activity was localized to the cytosolic fraction, and the procedures used to purify the *M. voltae* enzyme led to the preparation of pure enzyme, as judged by the results of SDS-PAGE analysis. The profile for the *M. jannaschii* enzyme is shown (Fig. 1), and similar results were obtained for the *M. thermolithotrophicus* and *M. igneus* enzymes (data not shown). We routinely observed a higher specific activity for the enzymes purified from *M. thermolithotrophicus*, *M. igneus*, and *M. jannaschii* than for that isolated from *M. voltae* (Table 1). Whether these differences indicate that the conditions of our standard assay fail to optimize the *M. voltae* enzyme is not known. The temperature-activity profile for each enzyme reflected the temperature for growth of the source methanogen (Table 1). For each enzyme, the temperature optimum was quite broad, and the results are reported as the temperature range within which the activity level was at least 80% of that observed at the optimum temperature.

Thermostability. The thermostability of the enzymes was determined by comparing the rate of their irreversible inactivation at both 68 and 89°C and extrapolating to the time required for 50% inactivation (Table 1). In general, the results reflect both the optimal temperature for enzymatic activity and the temperature for growth of each source organism. Nevertheless, we reproducibly observed that the *M. igneus* enzyme was more stable than the *M. jannaschii* enzyme and that the thermostability of the *M. jannaschii* and *M. thermolithotrophicus* enzymes did not differ significantly. As expected, the *M. voltae* enzyme was more stable at lower temperatures. For example, after incubation at 40 or 58.5°C for 1 h, the enzyme maintained 77.5 and 47% residual activity, respectively, compared with the enzyme stored on ice (data not shown). In considering these data, it is important to recognize that the degree of enzyme inactivation observed is very much dependent on the conditions of the assay. Thus, while the relative rates of enzyme inactivation do not vary, the degree of inactivation can differ by 20 to 30% depending on the ionic composition of the buffer solution or even the method used (e.g., incubation in polypropylene tubes in a temperature block versus stainless steel tubes in a forced-air oven).

Effect of KCl. The intracellular concentration of K^+ in *M. voltae* was reported to be approximately 725 mM (14). It was therefore of interest to examine the effects of potassium ions. For all four enzymes, potassium ions (added as the chloride) were stimulatory, and for the *M. igneus* and *M. thermolithotrophicus* enzymes, maximum stimulation was observed at a K^+ concentration of between 200 and 400 mM. In contrast, maximum stimulation of the *M. voltae* and *M. jannaschii* enzymes was observed at approximately 100 mM KCl. Sodium chloride was not stimulatory, indicating that neither Na^+ ions nor chloride stimulated the methanococcal enzyme. The stimulatory effect of potassium ions may reflect the requirement that the enzyme function in a cellular environment that is high in potassium ion concentration. It is also possible that potassium ions stabilize the enzyme at the temperatures of assay.

Source	Sequence
B. sub.	MNLVLM**GLPGAGKGT**QGERIVEDYGIPHISTGDMFRA
B. stea.	MNLVLM**GLPGAGKGT**QAEKIVAAYGIPHISTGDMFRA
E. coli	MRIILL**GAPGAGKGT**QAQFIMEKYGIPQISTGDMLRA
H. inf.	MNIILL**GAPGAGKGT**QAQFIMNKFGIPQISTGDMFRA
S. cer.	MSSSESIRMVLI**GPPGAGKGT**
H. sap.	MEEKLKKTKIIFVV**GGPGSGKGT**QCEKIVQKYGYTHLSTGDLLRS
C. carp.	ADKIKDAKIVFVV**GGPGSGKGT**QCEKIVEKYGYTHLSSGDLLRA
S. acid.	MKIGIVT**GIPGVGKST**VLAKVKEILDNQGINNKIINYGD
Mc. voltae	MKNKVVVVT**GVPGVG**STT**T**SXQLAMDNLRKEGVNYKMVS
Mc. thermo.	MKNKLVVVT**GVPGVG**GTT**T**IT
Mc. jann.	MKNKVVVIV**GVPGVG**STT**T**VXNKAIEELKKE
Mc. igneus	MKNKVVVIT**GVPGVG**GTT**T**XLQKTIEKLKEE

FIG. 2. N-terminal sequence comparison of adenylate kinases. Abbreviations: B. sub., *Bacillus subtilis*; B. stea., *Bacillus stearothermophilus*; E. coli, *Escherichia coli*; H. inf., *Haemophilus influenzae*; S. cer., *Saccharomyces cerevisiae*; H. sap., *Homo sapiens*; C. carp., *Cyprinus carpio* (common carp); S. acid., *Sulfolobus acidocaldarius*; Mc. voltae, *Methanococcus voltae*; Mc. thermo., *Methanococcus thermolithotrophicus*; Mc. jann, *Methanococcus jannaschii*; Mc. igneus, *Methanococcus igneus*. Except for the methanogen sequences, which were determined as part of this study, sequences were obtained from the GenBank or Swiss-Prot database. We have confirmed the reported sequence for the enzyme from *B. stearothermophilus* as a check on the reliability of our method. Amino acid residues that are present in all the proteins are shown in boldface type.

N-terminal sequence and amino acid composition. We determined the N-terminal sequence of each of the purified enzymes by Edman degradation (Fig. 2). From the sequence alignment, it is apparent that the enzymes have a region of similarity to the conserved "P-loop" region found in previously characterized adenylate kinases (25, 28). An exception is the absence of a lysine residue that is highly conserved in all reported adenylate kinases. In the case of the *M. voltae* adenylate kinase, the N-terminal sequence shown in Fig. 2 matches exactly the N-terminal sequence derived from the DNA sequence of the cloned gene (12a). It can also be seen that the five archeal sequences have a substitution for the usually conserved glycine residue that is adjacent to the conserved P-loop lysine. A comparison of the amino acid composition of each methanococcal enzyme showed that there was no obvious pattern of amino acid abundance that reflects the relative temperature optimum for enzyme activity (data not shown).

DISCUSSION

The characterization of enzymes from members of the Archaea, in particular the comparison of their structure with that of enzymes of identical or related function found in eukaryotes and bacteria, provides an opportunity to gain insights into the evolutionary relationships of enzymes as well as the strategies employed by thermophilic Archaea in their accommodation to life in extreme environments. With the recent identification of adenylate kinase in the archaeon *S. acidocaldarius* (16, 18) and our own isolation of this enzyme from a mesophilic and several related thermophilic methanogens, there is now the occasion to extend such comparisons to a ubiquitous enzyme for which there already exists much functional and structural information (26).

Since the purified enzymes catalyzed the conversion of ADP to ATP, an activity that was inhibited by both AMP and Ap_5A, they satisfy the general criterion for classification as an adenylate kinase. Furthermore, as estimated from SDS-PAGE

analysis, the molecular masses of the enzymes ranged from 23 to 25 kDa, and thus the enzymes are similar in size to all previously characterized adenylate kinases. Nevertheless, the phylogenetic relationship of the methanogen enzymes to members of the adenylate kinase family is uncertain and must await a determination of amino acid sequences. In this regard, it a noteworthy that the DNA-derived amino acid sequence of the *S. acidocaldarius* adenylate kinase (16) displayed only a low degree of sequence similarity to the characterized adenylate kinases of bacteria and eukaryotes except for a glycine-rich sequence close to the N terminus that matched well with the signature sequence, the phosphate-binding loop (P-loop), found in many nucleotide-binding proteins and in all characterized adenylate kinases (25, 28).

In adenylate kinases, the P-loop is located close to the N terminus, where it forms a loop between a β-strand and an α-helix (10, 12). The consensus sequence for the P-loop motif is GXXGXGKG(S)T, and the invariant lysine hydrogen bonds to the substrate phosphate (2, 22). A remarkable feature of the four methanogen adenylate kinases is that while each contains a sequence near the N terminus that exhibits striking similarity to the canonical P-loop sequence, the methanogen sequence lacks lysine. The lack of lysine within the very conserved P-loop region is not a general feature of archaeal adenylate kinases, since it is found in its expected position in the *S. acidocaldarius* enzyme (16, 18).

While it remains to be established if the glycine-rich sequence (GXXGXGXXT) found in these methanogens is indeed a nucleotide-binding peptide domain, both its presence near the N terminus and its similarity to the P-loop signature sequence raise interesting considerations. If the methanogen sequence is a nucleotide-binding motif, it is likely that the lack of lysine must be compensated in some way. One possibility is that nucleotide binding requires a positive charge contributed by a lysine residue but that the lysine residue resides outside the canonical P-loop region and participates in the binding interaction from another region of the chain. A similar situation in which a lysine from outside a glycine-rich nucleotide-binding domain is critical to ligand binding has been reported for a protein kinase (17). Alternatively, it is possible that the observed stimulation of methanogen adenylate kinase activity by potassium ions somehow reflects a lack of the usually invariant lysine residue and that potassium ion in some way compensates for the lack of positive charge provided by the peptide sequence. Clearly, future work must be directed towards studies that will resolve the structure of the methanogen adenylate kinase nucleotide-binding domains.

While our structural data are as yet quite limited, an interesting facet of our observations relates to the evolution of adenylate kinases. Since it is advantageous for all cells to have mechanisms to regulate energy metabolism through interconversions of the type catalyzed by adenylate kinases, it is expected that such an activity would be a feature of all cells. However, the question remains as to whether the ubiquity of adenylate kinase activity across the Bacteria, Eucarya, and Archaea reflects divergent or convergent evolution. In the case of the archaeon *S. acidocaldarius*, it has been proposed that its adenylate kinase may represent a novel enzyme class (16). In addition, our own limited data for the four methanogen enzymes suggest novel features of adenylate kinase structure not previously observed. Whether the adenylate kinases of the Archaea reflect an example of convergent evolution of enzyme function can only be addressed by additional functional and structural information. Of particular interest will be a survey of adenylate kinases from other methanogen and archaeal sources.

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