NAD kinase phosphorylates NAD\(^+\) to form NADP\(^+\). Conversely, NADP\(^+\) phosphatase, which has not yet been identified, dephosphorylates NADP\(^+\) to produce NAD\(^+\). Among the NAD kinase homologs, the primary structure of MJ0917 of hyperthermophilic archaeal \textit{Methanococcus jannaschii} is unique. MJ0917 possesses an NAD kinase homologous region in its C-terminal half and an inositol-1-phosphatase homologous region in its N-terminal half. In this study, MJ0917 was biochemically shown to possess both NAD kinase and phosphatase activities toward NADP\(^+\), NADPH, and fructose 1,6-bisphosphate, but not toward insitol 1-phosphate. With regard to the phosphatase activity, kinetic values indicated that NADP\(^+\) is the preferred substrate and that MJ0917 would function as a novel NADP phosphatase/NAD kinase showing conflicting dual activities, viz. synthesis and degradation of an essential NADP\(^+\). Furthermore, \textit{in vitro} analysis of MJ0917 showed that, although MJ0917 could supply NADP\(^+\), it prevented excess accumulation of NADP\(^+\); thus, it has the ability to maintain a high NAD\(^+\)/NADP\(^+\) ratio, whereas 5’-AMP would decrease this ratio. The evolutionary process during which MJ0917 arose is also discussed.

The functions of the well known molecules NAD\(^+\) and NADP\(^+\) are distinguishable. NAD\(^+\) is involved primarily in catabolic reactions, whereas NADP\(^+\) participates in anabolic reactions (1) and in defense against oxidative stress (2). NAD\(^+\) functions as a substrate for mono- and poly-ADP ribosylations and is involved in the formation of cyclic ADP-ribose (3). NADP\(^+\) is the substrate in the synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP\(^+\)) (3). Poly-ADP ribosylation has been implicated in the regulation of several processes, including DNA repair, transcription, and apoptosis, whereas both cyclic ADP-ribose and NAADP\(^+\) have been reported to participate in cytosolic calcium regulation in concert with inositol 1,4,5-trisphosphate (3). Thus, the intracellular balance of NAD\(^+\)/NADP\(^+\) appears to be important for a large number of cellular processes.

A key enzyme regulating the balance of NAD\(^+\)/NADP\(^+\) is NAD kinase (EC 2.7.1.23); it phosphorylates NAD\(^+\) to form NADP\(^+\). Another key enzyme involved in regulating this balance is NADP phosphatase (NADPase); it catalyzes the reverse reaction of NAD kinase, i.e. the dephosphorylation of NADP\(^+\) to NAD\(^+\). However, little information is available regarding NADPase because the enzyme has not yet been identified. NADPase activity has been detected in rat liver mitochondria (4) and in bacteria (5), and it was also found to correlate with the circadian rhythm of \textit{Euglena} (6) and with seed dormancy in \textit{Avena} sativa L. (7). Although NADPase activity has been detected in an outer membrane-associated acid phosphatase found in \textit{Helicobacter pylori} (8). In contrast to NADPase, NAD kinase has been biochemically characterized in various organisms such as Gram-positive bacteria (9, 10), Gram-negative bacteria (11, 12), yeast (2, 13, 14), plants (15), and humans (16); however, NAD kinase has not yet been reported in Archaea. Presently, >273 NAD kinase homologs are found in available data bases such as the Kyoto Encyclopedia of Genes and Genomes (available at www.genome.jp/kegg/).

Among the NAD kinase homologs found in the Kyoto Encyclopedia of Genes and Genomes, the primary structures of only two archaeal proteins (MJ0917 of \textit{Methanococcus jannaschii} and MMP1489 of \textit{Methanococcus maripaludis}) are characteristic and consist of two clearly distinguishable regions, viz. a C-terminal NAD kinase region and an N-terminal inositol-1-phosphatase (Ins-1-Pase) region (see Fig. 1A). \textit{M. jannaschii} and \textit{M. maripaludis} are hydrogenotrophic methanogenic Archaea whose entire genomic sequences have been determined (17, 18); they are thermophilic and mesophilic Archaea growing preferably at 85 and 37 \(\degree\mathrm{C}\), respectively (17, 19). Ins-1-Pase (EC 3.1.3.25) is known to dephosphorylate d-myo-inositol 1-phosphate, yielding myo-inositol, which is necessary for the synthesis of phosphatidylinositol (20).

A homolog (MJ0109) of the N-terminal region of MJ0917 (MJ0917-N) exists in \textit{M. jannaschii}, but not in \textit{M. maripaludis}. MJ0109 comprises 252 residues and has been shown to be a bifunctional Ins-1-Pase/fructose-1,6-bisphosphatase (Fru-1,6-Pase) (21, 22). Other archaeal MJ0917-N homologs (AF2372 of \textit{Archaeoglobus fulgidus} and TK0787 of \textit{Thermococcus kodakaraensis}) and a hyperthermophilic bacterial homolog (TM1415 of \textit{Thermotoga maritima}) have also been shown to be bifunctional Ins-1-Pases/Fru-1,6-Pases (22–25). The crystal structures of MJ0109 and AF2372 have been solved (22, 23).

The characteristic primary structures of MJ0917 and MMP1489, as well as the presence of Ins-1-Pase (MJ0109) in \textit{M. jannaschii}, raised questions about the function of both MJ0917 and MMP1489. We hypothesized that MJ0917 and MMP1489 may be novel bifunctional NADPases/NAD kinases with the potential to generate intracellular NAD\(^+\) and to maintain a suitable balance of NAD\(^+\)/NADP\(^+\). To confirm this hypothesis, we chose MJ0917 and analyzed its biochemical properties.
**Archaeal NADP Phosphatase/NAD Kinase**

**FIGURE 1. A, schematic representation of the primary structures of MJ0917 and MMP1489. MJ0917 and MMP1489 consist of 574 and 566 amino acid (a.a.) residues, respectively. The major regions of the N-terminal halves of MJ0917 (residues 2–298) and MMP1489 (residues 2–284) are detected as an Ins-1-Pase (E) and enzyme protein by Pafm (48), with E values of 3.5e-20 and 7.2e-25, respectively. The major regions of the C-terminal halves of MJ0917 (residues 303–561) and MMP1489 (residues 289–554) are detected as an NAD kinase by Pafm, with E values of 7.5e-105 and 4.3e-76, respectively.**

**Purification—** *E. coli* cells were suspended in TE buffer (10 mM Tris-HCl (pH 8.5) and 1.0 mM EDTA) at 4 °C and disrupted by sonication using an Insonator AF-Blue HS-650 M column (1.0 × 3.2 cm; Tosoh Corp., Tokyo) equilibrated with TE buffer containing 10 mM MgCl₂ at 4 °C and eluted with a linear gradient of NaCl (0–300 mM) in the same buffer (40 mL). The active fractions were combined and used as purified MJ0917. MJ0917-C was partially purified by heating the cell extract containing MJ0917-C at 85 °C for 2.5 min and then removing the aggregated proteins as described above.

**Other Analytical Methods—** SDS-PAGE was conducted with a 12.5% gel as described (29). Proteins in the gel were visualized with Coomassie Brilliant Blue R-250. The molecular mass of the enzyme was calculated by gel filtration chromatography on a Superdex 200 prep grade column (1.6 × 60 cm; Amersham Biosciences) with an AKTA purifier (Amersham Biosciences) as recommended by the manufacturer using 5.0 mM potassium phosphate (pH 7.0) containing 150 mM NaCl as the elution buffer and the standards in a high molecular weight calibration kit (Amersham Biosciences). To determine the N-terminal amino acid sequence, the purified enzyme was directly analyzed with a Procise 492 protein sequencing system (Applied Biosystems). The DNA sequence was determined using an automated DNA sequencer (Model 377, Applied Biosystems). BLAST homology analysis (30) was conducted on the GenomeNet web site (available at blast.genome.jp/) against the Kyoto Encyclopedia of Genes and Genomes (GENES) plus DGENES.
RESULTS

Expression and Purification—MJ0917 (full-length, residues 1–574), MJ0917-N (N-terminal half, residues 1–297), and MJ0917-C (C-terminal half, residues 298–574) were expressed in E. coli as recombinant proteins. SDS-PAGE of the E. coli cell extracts showed that MJ0917 (Fig. 1B, lane 3) and MJ0917-C (data not shown) were expressed as soluble proteins, but that MJ0917-N was expressed only as an insoluble protein (data not shown). The cell extract containing only the vector (pET-21b) did not show pNPPase or ATP-dependent NAD kinase activity, probably due to the high reaction temperature employed (85 °C). The cell extract containing soluble MJ0917 (full-length) showed both pNPPase and ATP-dependent NAD kinase activity, probably due to the high reaction temperature employed (85 °C). The cell extract containing soluble MJ0917 (full-length) showed both pNPPase and ATP-dependent NAD kinase activities (TABLE ONE). In contrast, the cell extract containing soluble MJ0917-C showed ATP-dependent NAD kinase activity, but no pNPPase activity (data not shown). Thus, MJ0917 was considered to be a fusion protein consisting of a phosphatase and an NAD kinase located in its N- and C-terminal halves, respectively. The pNPPase activity of MJ0917-N could not be confirmed because it could not be expressed as a soluble protein.

MJ0917 (full-length) was purified by measuring the activities of pNPPase and NAD kinase (TABLE ONE). Purified MJ0917 had a molecular mass of 64 kDa, which is in accordance with the calculated value (64,118 Da) (Fig. 1B). The N-terminal amino acid sequence of purified MJ0917 was determined as MVIMEGFK, which is the same as the N-terminal sequence deduced from the nucleotide sequence of the MJ0917 gene. Upon gel filtration chromatography, purified MJ0917 eluted as a single peak with an approximate molecular mass of 290 kDa, suggesting that MJ0917 is a homotetramer.

MJ0917 pNPPase and ATP-dependent NAD kinase activities were optimum under alkaline conditions (Fig. 2A) and were completely dependent on Mg2+. pNPPase and NAD kinase showed maximum activities at 20 and 50 mM Mg2+, respectively (Fig. 2B). MJ0917 pNPPase and ATP-dependent NAD kinase showed the highest activity at 100 °C and were inactive below 60 °C (Fig. 2C). M. jannaschii preferentially grows at 85 °C (17). Therefore, the phosphatase and NAD kinase activities of MJ0917 were hereafter determined at 85 °C in the presence of 20 mM Mg2+ at pH 8.5 unless stated otherwise.

Substrate Specificity of MJ0917—The substrate specificity (phosphoryl donor and acceptor) of MJ0917 NAD kinase was determined (TABLE TWO). As a phosphoryl donor, MJ0917 utilized nucleoside triphosphates and poly(P) as substrates, but not ADP or 5'-AMP, which is reminiscent of Mycobacterium tuberculosis NAD kinase (9). How-
ever, poly(P) is not considered to be the true phosphoryl donor in *M. jannaschii* because orthologs of the genes for poly(P)-synthesizing enzymes (poly(P) kinase-1 and -2), which occur in *M. tuberculosis*, are not found in *M. jannaschii* (31). MJ0917 used NADH as a phosphoryl acceptor but to a lesser extent, suggesting that NADH is not the physiological substrate.

The substrate specificity of the MJ0917 phosphatase was determined (TABLE THREE). MJ0917 exhibited high activity toward NADP⁺, NADPH, and 2'-AMP (analog of NADP⁺ and NADPH). Enzymatic formation of NAD⁺ from NADP⁺ was confirmed by TLC (data not shown). MJ0917 also showed high phosphatase activity toward fructose 1,6-bisphosphate (Fru-1,6-P₂). The phosphatase activities toward NADP⁺, NADPH, and Fru-1,6-P₂ were lower at pH 6.5 than at pH 8.5 (<10% activity) and preferred a high reaction temperature (optimum at 100 °C in a 1-min reaction). They also had an absolute requirement for Mg²⁺ (maximum activity at ~20 mM Mg²⁺). Partially purified MJ0917-C showed NAD kinase activity, but not NADPase activity, indicating that the NADPase activity of full-length MJ0917 is due to the phosphatase activity of the N-terminal Ins-1-Pase region (MJ0917-N) and not due to the reverse reaction of the C-terminal NAD kinase region (MJ0917-C).

The observation that MJ0917 did not show Ins-1-Pase activity was unexpected. The phosphatase was inert toward the substrates of NAD kinase, i.e., NAD⁺, NADH, ATP, and poly(P), although NADH and poly(P) are not supposed to be true substrates of NAD kinase as mentioned above. This demonstrates that the phosphatase activity of MJ0917 never interferes with the NAD kinase activity by degrading its substrates. The phosphatase in MJ0917 was also inactive toward ADP, 5'-AMP, and nicotinamide mononucleotide (NMN⁻). The inactivity of MJ0917 toward bis(p-nitrophenyl) phosphate, NAD⁺, NADH, and poly(P) also indicated that the enzyme was inactive in cleaving a phosphoester bond in diphosphate. Fru-1,6-Pase dephosphorylates the phosphate group at C-1 of Fru-1,6-P₂ (32). When considered collectively, it was concluded that the phosphatase in MJ0917 acts on the phosphate group at C-1 of Fru-1,6-P₂ and on the terminal phosphate group at C-2 of adenosine in 2'-AMP, NADP⁺, and NADPH, but not on the phosphate group at C-5 of adenosine in 5'-AMP, ADP, ATP, and at nicotinamide ribose in NMN⁺.

The NAD kinase activity of MJ0917 gave hyperbolic saturation curves for NAD⁺ and ATP, whereas the phosphatase activities exhibited sigmoidal kinetics toward NADP⁺, NADPH, and Fru-1,6-P₂ (Fig. 3). Kinetic values were determined (TABLE FOUR). NADP⁺, NADPH, and Fru-1,6-P₂ had *K₅₀* values of ~2.0, in good agreement with the positive cooperative sigmoidal behaviors of the phosphatase activities. The *K₅₀* value for the NAD kinase activity of NAD⁺ was higher than that of ATP and the *S₀.₅* values for the phosphatase activities. Among the kinetic values for the phosphatase activities toward NADP⁺, NADPH, and Fru-1,6-P₂, the *S₀.₅* and *K₅₀* values for NADP⁺ were the smallest and the highest, respectively. This indicates that NAD⁺ is the preferred substrate for the phosphatase in MJ0917 and that MJ0917 functions as a novel NADPase/NAD kinase exhibiting conflicting dual activities, viz. synthesis and degradation of NADP⁺.

**Inhibition of MJ0917**—The inhibitory effects of several compounds on MJ0917 NADPase, NADPHase, and Fru-1,6-Pase activities were examined (TABLE FIVE and supplemental Table 1S) at substrate concentrations (NADP⁺, NADPH, and Fru-1,6-P₂) that were similar to those of their *S₀.₅* values (0.2, 0.3, and 0.5 mM, respectively) (TABLE FOUR). The phosphate-containing compounds examined (NAD⁺, NADH, ATP, ADP, 5'-AMP, and NMN⁻) were resistant to the phosphatase activity of MJ0917 (TABLE THREE). Nicotinamide, NMN⁻, and adenosine exhibited little inhibitory effect (supplemental Table 1S). Among the other compounds, the inhibitory effects of 5'-AMP, NAD⁺, and ATP increased in the order 5'-AMP > NAD⁺ > ATP.

![Figure 3](https://example.com/figure3.png)

**TABLE THREE**

| Substrate                      | Relative activity |
|-------------------------------|-------------------|
| Ins-1-P                       | 0′′               |
| d-myo-Ins-2-P                 | 0′′               |
| Glycerol phosphate            | 11                |
| d-Glc-1-P                     | 0′′               |
| d-Glc-6-P                     | 0′′               |
| d-Fru-1-P                     | 0′′               |
| d-Fru-6-P                     | 0′′               |
| Fru-1,6-P₃                    | 432               |
| 5'-AMP                        | 0′′               |
| 2'-AMP                        | 500               |
| ADP                           | 0′′               |
| ATP                           | 0′′               |
| NAD⁺                          | 0′′               |
| NADP⁺                         | 574               |
| NADPH                         | 437               |
| NMN⁻                          | 0′′               |
| Bis(p-nitrophenyl) phosphate  | 0′′               |
| pNPP                          | 100               |
| Poly(P)                       | 0′′               |

* Activity was not detected (Aₓₓₓ < 0.01) at pH 6.5 (85 °C), 7.7 (85 °C), and 8.5 (85 °C) even when 0.125 units of MJ0917 was used in a 1-min reaction.
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**TABLE FOUR**

**Kinetic values of MJ0917 for NAD kinase and phosphatase activities**

The kinetic values are presented as the means ± S.D. for four (in the case of NAD kinase) and three (in the case of phosphatase) determinations.

| Compound | NADPase<sup>a</sup> | NAD kinase<sup>a</sup> |
|----------|---------------------|------------------------|
|          | %                   | %                      |
| None     | 100                 | 100                    |
| 5'-AMP   |                     |                        |
| 3.0 mM   | 11                  | 156                    |
| 1.0 mM   | 41                  | 128                    |
| 0.3 mM   | 61                  | 104                    |
| NAD<sup>+</sup> |           |                        |
| 3.0 mM   | 26                  |                        |
| 1.0 mM   | 53                  |                        |
| 0.3 mM   | 71                  |                        |
| ATP      |                     |                        |
| 3.0 mM   | 55                  |                        |
| 1.0 mM   | 79                  |                        |
| NADH     | 62                  |                        |
| 3.0 mM   | 79                  |                        |
| 1.0 mM   | 90                  |                        |
| ADP      |                     |                        |
| 3.0 mM   | 79                  | 71                     |
| 1.0 mM   | 84                  | 90                     |
| NADP<sup>+</sup> |     |                        |
| 0.3 mM   | 0                   |                         |
| 0.1 mM   | 60                  |                         |
| 0.03 mM  | 103                 |                         |
| 0.3 mM<sup>2</sup> | 63  |                        |
| 0.1 mM<sup>2</sup> | 74  |                        |
| NADPH    | 82                  |                         |
| 0.3 mM   | 97                  |                         |

<sup>a</sup> The NADPase and NAD kinase activities were assayed in the presence of NADP<sup>+</sup> at around its S<sub>0.5</sub> value (0.2 mM) and substrates at their respective K<sub>m</sub> values (3.0 mM for NAD<sup>+</sup> and 0.35 mM for ATP) as well as in the presence of the indicated compounds at the indicated concentrations. Each relative activity in the absence of the compound was taken as 100%.

<sup>b</sup> Activity was assayed in the presence of 3.0 mM NAD<sup>+</sup> and 3.0 mM ATP. The relative activity that was assayed under this condition without NADP<sup>+</sup> was taken as 100%.

ATP, whereas ADP and NADPH exhibited only a slight inhibitory effect (TABLE FIVE). The absence of inhibition by NMN<sup>+</sup> and adenosine, as well as the slight inhibitory effect of ADP, indicated that the structures of NAD<sup>+</sup> and 5'-AMP are important for inhibition. Of the phosphatase activities, Fru-1,6-Pase activity was the most sensitive to the inhibitory compounds (supplemental Table 1S). In the presence of both NAD<sup>+</sup> and ATP, a slight synergistic inhibitory effect was observed on Fru-1,6-Pase activity, but not on NADPase or NADPHase activity (supplemental TABLE 1S).

The effects of ADP, 5'-AMP, NADP<sup>+</sup>, and NADPH on MJ0917 NAD kinase activity were examined (TABLE FIVE) at substrate concentrations that were similar to those of their respective K<sub>m</sub> values (3.0 mM for NAD<sup>+</sup> and 0.35 mM for ATP) (TABLE FOUR). 5'-AMP activated the NAD kinase activity to a slight extent. ADP produced only a slight inhibitory effect, but NADPH produced no effect. Notably, even at 0.1 mM, NADP<sup>+</sup> produced an inhibitory effect, which was considerably greater at 0.3 mM; however, this inhibitory effect was relieved when the concentration of ATP was increased to 3.0 mM. This inhibitory effect of NADP<sup>+</sup> at a low ATP concentration (0.35 mM) and relief of this effect at a high ATP concentration (3.0 mM) were also observed with MJ0917-C, indicating that the ATP-dependent inhibitory effect of NADP<sup>+</sup> is a property of the MJ0917 NAD kinase region. However, the activating effect of 5'-AMP was not detected with MJ0917-C, implying that the activating effect of 5'-AMP observed with MJ0917 probably results from inhibition of the NADPase activity rather than activation of the NAD kinase activity.

With respect to the inhibitory effects of LiCl and NaCl on the phosphatase activities of MJ0917, the IC<sub>50</sub> values (concentrations required for 50% inhibition) of LiCl were 50 mM (NADPase), 30 mM (NAD-Phase), and 200 mM (Fru-1,6-Pase) and the IC<sub>50</sub> value of NaCl was 250 mM (Fru-1,6-Pase). NADPase and NADPHase activities were tolerant to NaCl. Even at 500 mM NaCl, 90 and 72% of the NADPase and NAD-Phase activities were retained, respectively. Lithium is a potent inhibitor of animal (bovine brain), yeast (Saccharomyces cerevisiae), plant, and bacterial (E. coli) Ins-1-Pases (IC<sub>50</sub> = 0.01–0.40 mM) (33–36), but not of Ins-1-Pases from Archaea (M. jannaschii MJ0109 and A. fulgidus AF2372) and hyperthermophilic bacterium (T. maritima TM1415) (IC<sub>50</sub> = 250, 290, and 100 mM, respectively) (21, 23, 25). Accordingly, the phosphatase activities of MJ0917 were resistant to LiCl. The phosphatase activities of MJ0917 were also tolerant to NaCl, as observed in the case of animal and plant Ins-1-Pases (34). Only the S. cerevisiae Ins-1-Pase is known to be slightly sensitive to NaCl (IC<sub>50</sub> = 80 mM) (34).

**Stoichiometry**—The biochemical data presented above were obtained by measuring the initial rate of production of NADP<sup>+</sup> (in the case of NAD kinase) or P<sub>i</sub> (in the case of phosphatase) in reactions that mostly lasted for up to 1 min. We also followed the reaction catalyzed by MJ0917 for longer incubation times by determining the amounts of NADP<sup>+</sup>, P<sub>i</sub>, and NAD<sup>+</sup> produced in the reaction mixture (Fig. 4, A and B, upper panels) or by TLC analysis (lower panels). After a 10-min incubation at 85 °C, ~75% of the input NAD<sup>+</sup> remained, whereas ATP was comparatively more stable (Fig. 4, A and B, lower panels, ±MJ0917). After a 15-min incubation, only ~50% of the NAD<sup>+</sup> remained (data not shown). Hence, we selected a reaction time of 10 min.
When 1.0 mM NAD\(^+\) and 1.0 mM ATP were used as substrates, both NADP\(^+\) and P\(_i\) were produced (Fig. 4A). During the initial period (until ~1 min), the amount of NADP\(^+\) was higher than that of P\(_i\), and the production rate of NADP\(^+\) was linear. However, during the additional incubation period, the amount of NADP\(^+\) gradually decreased, whereas that of P\(_i\), increased continuously. The amount of NAD\(^+\) decreased during the initial period (until ~1 min), but then was maintained at a high level during the remaining part of the incubation. TLC analysis showed that ATP was rapidly converted into ADP, whereas NAD\(^+\) remained comparatively unchanged (Fig. 4A). These behaviors of NADP\(^+\), P\(_i\), and NAD\(^+\), as well as those of ATP and ADP, were attributed to the dual activities of MJ0917: the NAD kinase activity that converts NAD\(^+\) into NADP\(^+\) by utilizing ATP and the NADPase activity that converts NADP\(^+\) into NAD\(^+\). The behaviors of NADP\(^+\), P\(_i\), and NAD\(^+\) were not significantly affected when the substrate concentrations were increased (3.0 mM NAD\(^+\) and 3.0 mM ATP) (Fig. 4B), although the levels of both NADP\(^+\) and P\(_i\) were higher.

The effects of 5'-AMP were examined in the presence of 1.0 mM NAD\(^+\) and 1.0 mM ATP (Fig. 4C). 5'-AMP suppressed the production of P\(_i\) and increased the production of NADP\(^+\) during incubation; this demonstrated that the activating effect of 5'-AMP is due to the suppression of the NADPase activity (TABLE V) rather than activation of the NAD kinase activity.

Homology and Cluster Analysis—A BLAST homology search was conducted using the primary structures of MJ0917-N (residues 1–297) and MJ0917-C (residues 298–574) as queries. MJ0917-N detected 314 homologs that were chiefly annotated as Ins-1-Pases (E-value < 1.0) and 314 homologs that were chiefly annotated as Ins-1-Pases (E-value < 6e-05). Some of the BLAST search results are shown in supplemental TABLE 2S. MJ0917-N and MJ0917-C showed the highest homology to M. maripaludis MMP1489, which is also a fusion protein of an Ins-1-Pase and an NAD kinase (Fig. 1A), and higher homology to 10 and 6 proteins, respectively, from Archaea (Euryarchaeota), to which M. jannaschii and M. maripaludis belong. The functions of some of the MJ0917-N homologs have been biochemically confirmed, e.g. Ins-1-Pase, Ins-1-Pase/Fru-1,6-Pase, and Fru-1,6-Pase (supplemental Table 2S). MJ0917-N and MJ0917-C showed the highest homology to M. maripaludis MMP1489, which is also a fusion protein of an Ins-1-Pase and an NAD kinase (Fig. 1A), and higher homology to 10 and 6 proteins, respectively, from Archaea (Euryarchaeota), to which M. jannaschii and M. maripaludis belong. The functions of some of the MJ0917-N homologs have been biochemically confirmed, e.g. Ins-1-Pase, Ins-1-Pase/Fru-1,6-Pase, and Fru-1,6-Pase (supplemental Table 2S).

Cluster analysis, which detects operon-like structures, for each of the 314 and 273 orthologs of MJ0917-N and MJ0917-C classified them into 57 and 41 cluster groups, respectively. (Cluster groups were not observed in the 71 and 48 orthologs of MJ0917-N and MJ0917-C.) Of the cluster groups, only orthologs of MJ0917-N and MJ0917-C in Euryarchaeota (Methanobacterium thermoautotrophicum, Methanosarcina acetivorans, Methanosarcina mazei, A. fulgidus, and Methanopyrus kandleri) form a cluster, i.e. the phosphatase ortholog and the NAD kinase ortholog in these Euryarchaeota form an operon-like structure (Fig. 5).

DISCUSSION

In this study, MJ0917 was biochemically shown to possess both NAD kinase and phosphatase activities toward NADP\(^+\), NADPH, and Fru-
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1,6-P₃. However, MJ0917 did not exhibit Ins-1-Pase activity. This suggests that the other uncharacterized MJ0917-N (Ins-1-Pase) homologs may also not exhibit Ins-1-Pase activity, but may show another kind of activity (e.g. NADPase or Fru-1,6-Pase); it may also imply that the other characterized Ins-1-Pases exhibit NADPase activity. Although several Ins-1-Pases have been characterized, to our knowledge, NADPase activity has not received any attention (21–25, 34, 36, 40). Exceptions are the Ins-1-Pases found in *M. tuberculosis* and rat brain, which show <3 and 11% NADPase activity, respectively, compared with their Ins-1-Pase activities (39, 42).

NADPase, NADPHase, and Fru-1,6-Pase activities exhibited positive cooperative sigmoidal behaviors (Fig. 3) that were in good agreement with the *nₛ* values (~2.0) for NADP⁺, NADPH, and Fru-1,6-P₃ (TABLE FOUR). The crystal structures of MJ0109 and AF2372 (Ins-1-Pase/Fru-1,6-Pases, homologs of MJ0917-N) are dimers and contain one substrate-binding site/subunit (22, 23); this implies that one substrate-binding site exists in one molecule of the MJ0917-N portion. Based on this, it is possible that two substrate molecules bind in a cooperative manner to the two MJ0917-N portions in tetrameric MJ0917, enabling sigmoidal kinetics with *nₛ* values of ~2.0.

The fact that MJ0917 showed both NAD kinase and NADPase activities, whereas NADP⁺ was considered to be the most preferred substrate based on kinetic values (TABLE FOUR), indicates that MJ0917 would function as a novel NADPase/NAD kinase with conflicting dual activities, viz. synthesis and degradation of NADP⁺. It should be noted that NADPase had not yet been identified. MJ0917 is the sole NAD kinase ortholog found in *M. jannaschii*, and NAD kinase is reported to be essential in *M. tuberculosis* and *Bacillus subtilis* (43, 44). How can essential NADP⁺ be supplied by MJ0917 in *M. jannaschii?* The *in vitro* analysis of MJ0917 not only answered this question, but also suggested that only MJ0917 has the ability to prevent excess NADP⁺ accumulation, thereby maintaining a high NAD⁺/NADP⁺ ratio. NADP⁺ is produced by MJ0917, but never accumulates continuously to excess levels, resulting in low NAD⁺ and high NAD⁺ levels (Fig. 4, A and B). NADPase activity, as well as the product inhibition of NAD kinase activity at a low ATP level (TABLE FIVE), should contribute to preventing excess NADP⁺ accumulation. It should be noted that, during the initial reaction period, the production rate of NADP⁺ was higher than that of P₃ (the conversion rate of NADP⁺ into NAD⁺) (Fig. 4, A and B). The sigmoidal kinetics of the NADPase reaction (Fig. 3) and the inhibitory effects of substrates (NAD⁺ and ATP) of the NAD kinase reaction on the NADPase reaction (TABLE FIVE) should contribute to this lower production rate of P₃. Furthermore, 5'-AMP suppressed the NADPase reaction (TABLE FIVE) and allowed MJ0917 to produce higher levels of NAD⁺, thus decreasing the NAD⁺/NADP⁺ ratio (Fig. 4C) and suggesting that 5'-AMP regulates the ratio of NAD⁺ to NADP⁺. Despite the suppressed production rate of P₃, it might be supposed that NADP⁺ should be completely depleted through a continuous conversion of NADP⁺ into NAD⁺ (Fig. 4). However, NADP⁺ would not be completely depleted if it were assumed that ATP is supplied continuously *in vivo* because a sufficient amount of NAD⁺ would be produced due to the NADPase reaction. Collectively, MJ0917 would maintain a high NAD⁺/NADP⁺ ratio, wherein 5'-AMP would act to decrease the ratio. The NAD⁺/NADP⁺ ratio in living cells is regarded as being high, e.g. the ratio is 5.3 in *E. coli* and 8.3 in *S. cerevisiae* (45, 46). Although the physiological NAD⁺/NADP⁺ ratio in archaecal cells has not been reported, the MJ0917 NADPase reaction would be advantageous in maintaining a high ratio in *M. jannaschii*. To maintain a high ratio in other cells, a high NADPase activity and/or sufficient inhibition of NAD kinase activity should be required. However, no potent inhibitors for NAD kinase activity have yet been found among biochemically characterized NAD kinases (with the exception of *E. coli* NAD kinase, which is strongly inhibited by NADH and NADPH) (2, 9–16). Hence, we propose that the NADPase reaction in other cells should also prevent the excess accumulation of NADP⁺ and should help maintain a high NAD⁺/NADP⁺ ratio.

The NADPase and Fru-1,6-Pase activities of MJ0917 might suggest that MJ0917 functions physiologically as a NADPase and a Fru-1,6-Pase. However, the physiological function of the Fru-1,6-Pase activity has remained questionable because it was genetically demonstrated that TK0787 (the MJ0917-N homolog) of *T. kodakaraensis* is not a "true" Fru-1,6-Pase (24, 47). The true Fru-1,6-Pase of *T. kodakaraensis* is TK2164, and its ortholog in *M. jannaschii* is MJ0299 (24, 47). Stec et al. (22) hypothesized that MJ0109 (Ins-1-Pase/Fru-1,6-Pase) is an archetypal cyclitol phosphatase from which the more substrate-specific Ins-1-Pase and Fru-1,6-Pase of eubacteria and eucaryotes arose by divergent evolution. We favor their hypothesis and also assume that MJ0917-N represents the evolutionary origin of the more substrate-specific NADPases, NADPHases, and Fru-1,6-Pases of eubacteria and eucaryotes, although the more substrate-specific NADPases and NADPHases of eubacteria and eucaryotes have not yet been identified.

How did MJ0917 or MMP1489 arise during the evolutionary process? Cluster analysis provided insights into this question (Fig. 5); with the exception of the clustered orthologs of *M. acetivorans*, the 3'-regions of the clustered MJ0917-N (phosphatase) orthologs overlap with the 5'-regions of the MJ0917-C (NAD kinase) orthologs, thereby resulting in different reading frames. In the orthologs (41 bases overlapping) of *M. mazei*, an insertion of 2 bases or a deletion of 1 base from a region around the junction of the two orthologs can easily result in the fusion of the two orthologs. In the other orthologs (4 or 28 bases overlapping), an insertion of 1 base or a deletion of 2 bases can also result in the fusion of the two orthologs. Thus, we suggest that MJ0917 and MMP1489 could have been generated through an overlap of the two genes, which still occur separately in the present genomic sequences of several Eurarchaea shown in Fig. 5. In this context, we also propose that at least the clustered MJ0917-N orthologs should be NADPase genes.

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