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Nucleoside diphosphate kinase from haloalkaliphilic archaeon *Natronobacterium magadii*: purification and characterization

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Abstract An ATP-binding protein from the haloalkaliphilic archaeon *Natronobacterium magadii* was purified and characterized by affinity chromatography on ATP-agarose and by fast protein liquid chromatography (FPLC) on a Mono Q column. The N-terminal 20 amino acid sequence of the kinase showed a strong sequence similarity of this protein with nucleoside diphosphate (NDP) kinases from different organisms and, accordingly, we believe that this protein is a nucleoside diphosphate kinase, an enzyme whose main function is to exchange γ-phosphates between nucleoside triphosphates and diphosphates. Comparison of the molecular weights of the NDP kinase monomer determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (23000) and of the oligomer determined by sedimentation equilibrium experiments (125000) indicated that the oligomer is a hexamer. The enzyme was autophosphorylated in the presence of [γ-^{32}P]ATP, and Mg^{2+} was required for the incorporation of phosphate. The kinase preserved the ability to transfer γ-phosphate from ATP to GDP in the range of NaCl concentration from 90 mM to 3.5 M and in the range of pH from 5 to 12. It was found and confirmed by Western blotting that this kinase is one of the proteins that bind specifically to natronobacterial flagellins. NDP kinase from haloalkaliphiles appeared to be simple to purify and to be a suitable enzyme for studies of structure and stability compared with NDP kinases from mesophilic organisms.

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Introduction

Nucleoside diphosphate (NDP) kinases are proteins with molecular mass of 17–22 kDa that form oligomeric complexes. Eukaryotic NDP kinases are usually hexamers (Chiadmi et al. 1993) and bacterial ones are tetramers (Williams et al. 1993; Almaula et al. 1995). As a rule the oligomers are composed of one type of subunit, but in cells of mammalian tissues, oligomers may be composed of two types of highly homologous NDP kinase subunits (Guignard and Markert 1996). Not only cytosolic, but also membrane-associated, NDP kinases have been described (Kimura and Shimada 1988). The main function of NDP kinases is to exchange γ-phosphates between nucleoside triphosphates and diphosphates (Uesaka et al. 1987). The mechanism of enzymatic reaction involves formation of a phosphorylated intermediate by phosphorylation of a histidine residue at the active site of the NDP kinase (Deville-Bonne et al. 1996). NDP kinases are able to form complexes with GTP-dependent proteins (Uesaka et al. 1987; Nickerson and Wells 1984; Biggs et al. 1990). They can act as protein kinases to phosphorylate other proteins using ATP or GTP (Lu et al. 1996; Inoue et al. 1996). NDP kinases can specifically bind DNA (Hildebrandt et al. 1995). It is supposed that NDP kinases are involved in development, growth control, and oncogenic transformation (Wallet et al. 1990).

NDP kinases have been found in the cells of many organisms, although this protein has never been described in the Archaea. However, recently, the gene encoding NDP kinase was found in the *Methanococcus jannaschii* genome (Bult et al. 1996). In this paper we describe the purification and characterization of a putative NDP kinase from the haloalkaliphilic archaeon *Natronobacterium magadii* (optimal growth conditions: 4 M NaCl, pH 9.5, 45°C).

Materials and methods

Strains and growth conditions

Natronobacterium magadii DSM-3394 and *Natronobacterium pharaonis* DSM-2160 (obtained from Deutsche Sammlunge von Mikroorganismen, Gottingen, Germany) were grown as described (Polosina et al. 1996).

NDP kinase purification

Affinity chromatography. Columns (5 ml) with 1 ml of ATP-agarose (Sigma, St. Louis, Mo, USA) and flagellin-Sepharose were used. To obtain flagellin-Sepharose, natronobacterial flagellins were coupled to CNBr-Sepharose according to Kostyukova et al. (1994). To obtain a cytoplasmic extract, natronobacterial cells were pelleted at 10000*g* for 20 min and resuspended in buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing 3.5 M NaCl. The cells were broken by sonication (22 kHz, 2 min) and cell envelopes were pelleted by centrifugation at 15000*g* for 15 min. The supernatant was centrifuged at 35000*g* for 1 h and loaded onto a column with ATP-agarose or flagellin-Sepharose. To remove nonadsorbed material, the gel was washed with 6 volumes of buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing 3.5 M NaCl. Proteins bound to gel were eluted by adding 5 mM ATP to the buffer, or by the buffer, without NaCl and KCl.

Ion exchange chromatography. After affinity chromatography, NDP kinase was purified from the remaining contaminating proteins by fast protein liquid chromatography (FPLC). Proteins were transferred to 20 mM Tris-HCl, pH 8.0 and fractionation of proteins was done on an anion-exchange Mono Q column by gradient elution (0.2–0.85 M NaCl) in 20 mM Tris-HCl, pH 8.0). The purity of the kinase was checked electrophoretically. After purification the kinase was dialyzed against buffers containing different NaCl concentrations. The kinase concentration was determined by the biuret and Lowry procedures using a kit for microdetermination of the total protein (Sigma) with bovine albumin as a standard.

N-terminal sequence determination

Protein bands selected for sequencing were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) by the method of Towbin et al. (1979) and sequenced with a pulsed-liquid phase sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed by the Core Facility for DNA/Protein Chemistry, Department of Biochemistry, Queen's University, Kingston, ON, Canada.

Determination of holoenzyme molecular weight by equilibrium sedimentation

For equilibrium sedimentation experiments, 0.3 ml aliquots of NDP kinase solution in buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing different concentrations of NaCl (450 mM, 900 mM, 1.75 M, and 3.5 M) were used. The kinase concentration was 0.25–0.3 mg/ml. Experiments were done using an analytical centrifuge MOM 3170 (Hungary) equipped with interference optics. Samples were centrifuged at 28400 rpm at 20°C. Calculations were done according to Yphantis (1964).

Limited proteolysis

Limited proteolysis by trypsin was done in the buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing different concentrations of NaCl (from 0 to 3.5 M) and by thermolysin at various temperatures (from 20° to 80°C). The NDP kinase/protease ratio was 20:1. Aliquots for electrophoresis were taken at definite time periods. Proteolysis was stopped by adding sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol, 2.3% SDS) and boiling.

Phosphorylation experiments

The kinase was incubated in different salt and pH conditions with 30 μCi [γ-^{32}P]ATP for 40 min at room temperature in the presence or absence of 5 mM GDP. To check NDP kinase enzymatic activity in different salt and pH conditions, the following buffers were used: 20 mM Tris-HCl, pH 8.0, containing 90 mM, 180 mM, 450 mM, 900 mM, 1.75 M, and 3.5 M NaCl; 20 mM CH_3COONa, pH 4.0 and 5.0, containing 1.75 M NaCl; 20 mM KH_2PO_4, pH 6.0, 1.75 M NaCl; 20 mM Tris-HCl, pH 6.0, 7.0, 8.0, 9.0, and 10.0, containing 1.75 M NaCl; 20 mM Na_2HPO_4, pH 11.0, 1.75 M NaCl; 0.5 M KCl/NaOH, pH 12.0, 1.75 M NaCl; 0.5 M KCl/NaOH, pH 13.0, 1.75 M NaCl. For the experiments on K^+ and Mg^{2+} -dependence, 10 mM KCl and 10 mM $MgCl_2$ were used. To remove Mg^{2+} from the reaction mixture, 100 mM ethylenediaminetetraacetic acid (EDTA) was added before incubation. After incubation, SDS-PAGE sample buffer was added to the reaction mixture and the samples were analyzed by SDS-PAGE followed by autoradiography. The film was exposed for 24 h at −20°C and developed as described in the manufacturer's specification.

SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method of Laemmli (1970) using 12% acrylamide gels. Protein molecular mass markers of 212, 158, 116, 97, 66.4, 55.5, 42.7, 36.5, 26.6, 20.1, 14, 6.5 kDa (Biolabs, USA) and 97.4, 66.2, 45, 31, 21.5, 14.4 kDa (Bio-Rad, Hercules, CA,

USA) were used. For immunostaining, proteins were blotted from gels onto nitrocellulose membranes (Sigma) at 25 V for 2 h using the buffer described by Towbin et al. (1979). The blots were developed using, as primary antibodies, a 1:250 dilution of antiserum raised in rabbits to the purified *N.magadii* NDP kinase. Bound antibodies were detected using a kit (Promega, Madison, WI, USA) with alkaline phosphatase conjugated antibodies at a dilution 1:5000 and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as the color development substrates.

Results and discussion

A cytosolic protein with a molecular weight of 23000 that can bind ATP was found in cells of the haloalkaliphilic archaea *Natronobacterium magadii* and *Natronobacterium pharaonis* (Kostyukova et al. 1994). This protein was obtained as a major protein that bound to ATP-agarose and was eluted by both ATP and GTP.

The protein was purified from the contaminating proteins (Fig. 1) and its 20 N-terminal amino acids were determined. The comparison with known sequences showed a strong sequence similarity between this protein and all known nucleoside diphosphate kinases from different organisms (Fig. 2). No homology was found with other proteins.

Analytical centrifugation was used to check the ability of the purified kinase to form oligomeric structures. It was found that in different NaCl concentrations (from 3.5 M to 450 mM) the kinase exists as a complex. Based on the calculations, the average molecular weight of the complex is 125000 ± 5000. By comparison of the molecular weight of the NDP kinase monomer determined by SDS-PAGE (23000 ± 2000) and that of the oligomer determined by sedimentation equilibrium experiments, the oligomer consists of 5.5 ± 0.3 subunits, and this indicates that in all probability it is not a tetramer but a hexamer and therefore it is similar to eukaryotic NDP kinases.

To study changes in the kinase stability under decreasing NaCl concentration and increasing temperature, the protein was treated correspondingly by trypsin at different NaCl concentrations (0, 0.45, 0.9, 1.35, 1.75, and 3.5 M) at room temperature and by thermolysin at different temperatures (20°, 45°, 60°, and 80°C) at a NaCl concentration of 1.75 M. It was shown that the natronobacterial NDP kinase is very stable to protease treatment in a range of NaCl concentrations from 1.75 M to 3.5 M and temperatures up to 65°C. The enzyme was digested rapidly after thermal treatment up to 80°C or decreasing the NaCl concentration down to zero (Fig. 3). The fact that the kinase lost its stability to proteases not only under thermal treatment, but also after decreasing the salt concentration, indicates that the protein denatures at a low salt concentration. Stability to proteases was restored completely after cooling or during dialysis against buffers containing higher NaCl concentrations in the presence of ATP.

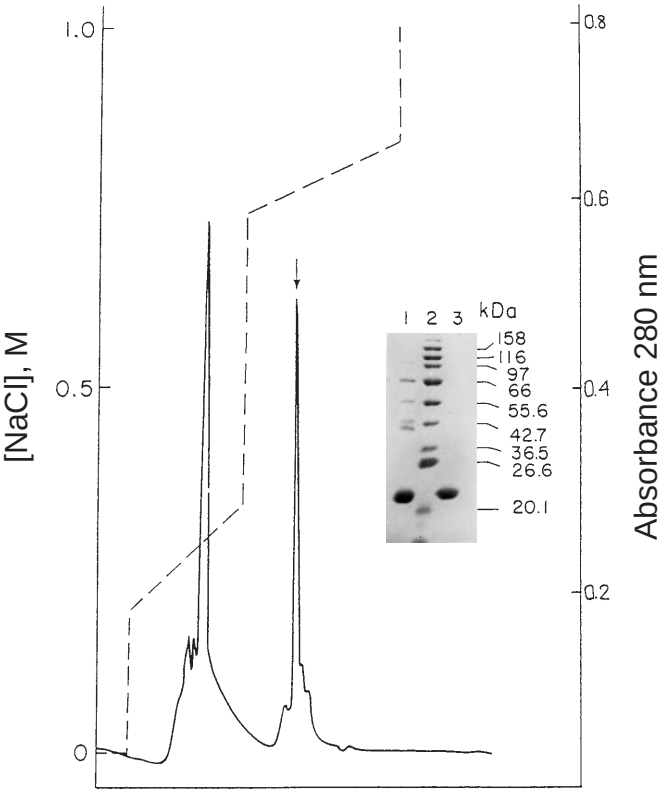

Fig. 1. Elution profile obtained after fast protein liquid chromatography (FPLC) of ATP-binding proteins. *Solid line*, absorbance at 280 nm; *broken line*, NaCl concentration. *Arrow* indicates the kinase-containing fraction of column eluates. The inset shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins eluted from ATP-agarose (*lane 1*), molecular weight standards (*lane 2*), and the purified kinase (*lane 3*)

Solutions of purified kinase had a typical nucleotide UV spectrum with a maximum near 260 nm, indicating that the kinase binds ATP very strongly and retains the ATP even after dialysis against buffer without ATP and with a low NaCl concentration (0–180 mM). It was possible to obtain the kinase in an ATP-free state by using buffer without NaCl and KCl for elution from the ATP-agarose. In this case, the amount of contaminating protein increased but the UV spectrum of the purified NDP kinase looked like a normal protein spectrum. Adding 5 mM ATP into the extract did not influence the kinase binding to ATP-agarose but resulted in a substantial decrease in the binding of contaminating proteins. Removal of Mg^{2+} from the extract by adding 100 mM EDTA did not influence the kinase yield, showing that the ability of the kinase to bind ATP does not depend on the presence of Mg^{2+}. It was also shown that the kinase could be eluted from ATP-agarose by the buffer containing ADP or AMP, whereas other proteins were eluted. Elution of the kinase was achieved by adding GTP to the buffer. These experiments indicate the possibility of purifying the NDP kinase in one chromatography step without transfer into low salt conditions where denaturation may occur.

Fig. 2. Comparison of N-terminal amino acid sequences of *Natronobacterium magadii* kinase and nucleoside diphosphate kinases from *Methanococcus jannaschii* (Bult et al. 1996), *Drosophila melanogaster* (Biggs et al. 1988), *Escherichia coli* (Hama et al. 1991), *Bacillus subtilis* (Henner et al. 1985), *Rattus norvegicus* (Kimura et al. 1990), *Homo sapiens* (Rosengard et al. 1989), *Dictyostelium discoideum* (Lacombe et al. 1990). Identical amino acids (*bold*)

S E H **E R T F V M V** X **P D A** F A **R G L V G**	*Natronobacterium magadii*
M K **E R T F V** A L K **P D A** V K **R** K **L** I **G**	*Methanococcus jannaschii*
E R T F I **M V** K **P D** G V Q **R G L V G**	*Drosophila melanogaster*
A I **E R T F** S I I K **P** N **A** V **A** K N **V** I **G**	*Escherichia coli*
M M **E** K **T F** I **M V** K **P D** G V Q **R** Q **L** I **G**	*Bacillus subtilis*
A N L **E R T F** I A I K **P D** G V Q **R G L**	*Rattus norvegicus*
M A N C **E R T F** I A I K **P D** G V Q **R G L**	*Homo sapiens*
M S T N K V N K **E R T F** L A **V** K **P D G V**	*Dictyostelium discoideum*

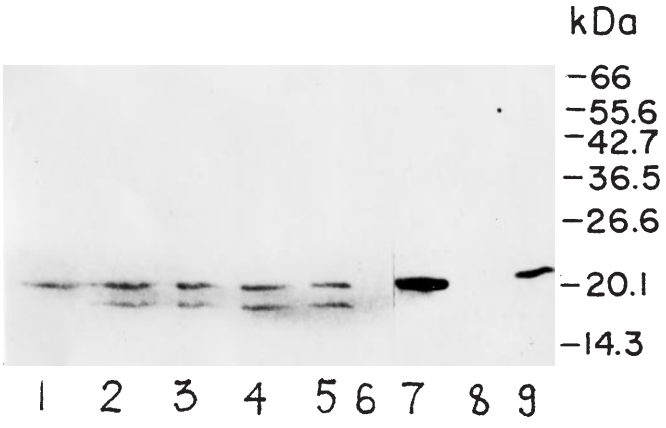

Fig. 3. Results of cleavage of NDP kinase from *N.magadii* by trypsin (*lanes 1–6*) and thermolysin (*lanes 7–9*) in the buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing 5 mM ATP and various NaCl concentrations. The NDP kinase/protease ratio was 20:1. Purified kinase (*lane 1*); the kinase treated by trypsin (duration of proteolysis 1 min) in 1.75 M (*lane 2*), 1.35 M (*lane 3*), 900 mM (*lane 4*), 450 mM (*lane 5*), and 0 (*lane 6*) NaCl; the kinase in 1.75 M NaCl treated by thermolysin (duration of proteolysis 3 min) before heating (*lane 7*), at 80°C (*lane 8*), after cooling (*lane 9*). The *right-hand scale* shows molecular mass standards

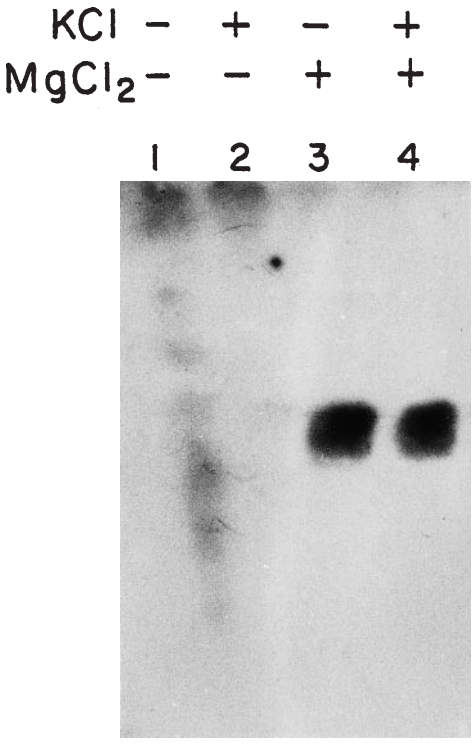

Fig. 4. Autoradiograph of SDS-PAGE with results of phosphorylation experiments. NDP kinase in 20 mM Tris-HCl, pH 8.0 containing 900 mM NaCl (*lanes 1–4*) was treated with [γ-^{32}P]ATP in the presence or absence of 10 mM KCl and 10 mM $MgCl_2$

The NDP kinase in an ATP-free state was obtained and purified in two chromatography steps to check its enzymatic activity in different salt conditions. It was shown that the protein bands obtained after SDS gel-electrophoresis of samples containing [γ-^{32}P]ATP had a radioactive label (Fig. 4) that indicates formation of a phosphorylated intermediate at the first step. In experiments on the influence of K^+ and Mg^{2+}, it was shown that Mg^{2+} was required for the kinase autophosphorylation (Fig. 4). Without adding EDTA, the kinase autophosphorylated, even after dialysis in buffer without Mg^{2+}. This means that the kinase binds Mg^{2+} very strongly. These data differ from those obtained for other NDP kinases where the phosphotransfer reaction from ATP to the enzyme can occur without Mg^{2+} (Williams et al. 1993). Bands in samples containing [γ-^{32}P]ATP had no radioactive label after adding GDP, since at the second step $^{32}P_i$ was transferred from the kinase to GDP (Fig. 5). It was also found that the ability of phosphorylated kinase to bind to ATP-agarose decreased several fold.

It was shown that the kinase preserved the ability to transfer γ-phosphate from ATP to GDP in the range of NaCl concentration from 90 mM to 3.5 M and in the pH range from 5.0 to 12.0. However, the kinase enzymatic ac-

Fig. 5. Autoradiograph of SDS-PAGE with results of phosphorylation experiments. NDP kinase in 20 mM Tris-HCl, pH 8.0 containing 3.5 M NaCl was treated with [γ-^{32}P]ATP with GDP (*lane 1*) and without GDP (*lane 2*)

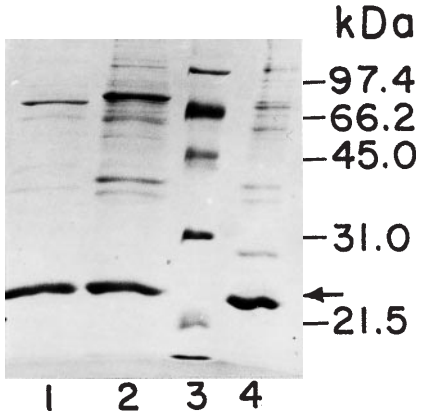

Fig. 6. SDS-PAGE of proteins from *N.magadii* (*lanes 1*, *4*) and *N.pharaonis* (*lane 2*) cytoplasmic extracts eluted from ATP-agarose (*lanes 1*, *2*) or natronobacterial flagellin-Sepharose (*lane 4*) by buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) containing 5 mM ATP. Molecular weight standards are in *lane 3*. The *arrow* indicates the kinase

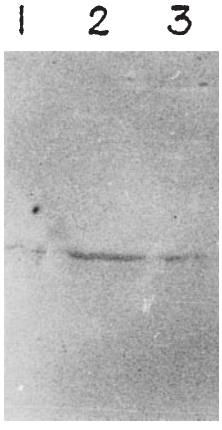

Fig. 7. Western blot of proteins from *N.pharaonis* (*lane 1*) and *N.magadii* (*lane 2*) obtained on ATP-agarose; and proteins from *N.magadii* obtained on flagellin-Sepharose (*lane 3*) with antiserum to the purified kinase from *N.magadii*

tivity decreased in NaCl concentrations of 180 mM and lower and decreased at least two-fold at pH values 5.0 and 6.0. A phosphorylated intermediate was not formed in buffers without salt or in buffers of pH 4.0 or 13.0. It was shown that the protein preserved activity after heating up to 90°C with subsequent cooling. The ability to restore enzymatic activity and stability to proteases after thermal treatment distinguishes natronobacterial kinase from the eukaryotic and bacterial NDP kinases that denature irreversibly after heating at 70°C (Giartosio et al. 1996).

Antiserum was obtained against the kinase from *N.magadii*. The protein from *N.pharaonis* obtained by the same procedure (Fig. 6, lane 2), the *N.magadii* proteins obtained on flagellin-Sepharose (Fig. 6, lane 4), and cell lysates of both natronobacteria were checked by Western blotting. There was good reaction of the antiserum with purified kinases from both *N.magadii* and *N.pharaonis* (Fig. 7) and there was a faint band of the same molecular weight in cell lysates that crossreacted with the antiserum (data not shown). This indicates that the kinase is a minor protein and only its strong affinity for ATP makes it a major component among the proteins that bind to ATP-agarose. This observation, and the fact that the NDP kinase may be obtained without ATP bound, even when ATP is added to the extract, indicate that it exists in the cell mainly in association with other proteins and that NDP/NTP exchange occurs very quickly.

It is known that NDP kinases are able to associate with other proteins, mainly GDP/GTP-binding proteins. NDP kinase was found associated with microtubules (Nickerson and Wells 1984; Biggs et al. 1990), and with the Hsc70 molecular chaperone where the kinase functions as an accessory protein (Leung and Hightower 1997). It was found and confirmed by Western blotting that this kinase is one of the proteins that bind specifically to natronobacterial flagellins (Fig. 5, lane 4). As no GTP activity was found for flagellins, this may mean that NDP kinase associates with another GTP-dependent protein that is able to bind to flagellins. Another explanation of the NDP kinase binding with flagellins is connected with its possible protein kinase activity. It was found that NDP kinases are able to phosphorylate other histidine protein kinases, e.g., CheA (Lu et al. 1996), or proteins, e.g., ovalbumin (Inoue et al. 1996). Natronobacterial flagellins are stained blue like phosphorylated proteins by Stains-All (Pyatibratov et al. 1996) and it is the NDP kinase that evidently phosphorylates them.

The Archaea is a domain that includes the extremely anaerobic methanogens, halophiles, and thermoacidophiles. Most representatives of this domain live at extreme pH, temperature, or salt concentration. Enzymes from these microorganisms have to possess increased stability to be able to function under such extreme conditions. NDP kinase from haloalkaliphiles appears to be easily purified,

and could be a suitable enzyme for studying structure and stability and comparing these data with those for NDP kinases from mesophilic organisms.

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