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Stability of *Natrialba magadii* NDP kinase: comparisons with other halophilic proteins

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Abstract Nucleoside diphosphate kinase from the haloalkaliphilic archaeon *Natrialba magadii* (*Nm* NDPK) is a homooligomeric hexamer with a monomer molecular weight of 23 kDa. Its main function is to exchange γ-phosphates between nucleoside triphosphates and diphosphates. Previously it was shown that *Nm* NDPK is active over a wide range of NaCl concentrations, which is not typical of extremely halophilic proteins. In this paper more detailed investigations of kinase function and stability were carried out using circular dichroism, differential scanning calorimetry, size-exclusion chromatography, and biochemical methods. A possible mechanism for stabilization of halophilic proteins that allows them to function in a wide range of NaCl concentrations is proposed.

Key words Archaea · Differential scanning calorimetry · Haloalkaliphiles · Nucleoside diphosphate kinase · Stability

Introduction

The halophilic archaea are a group of organisms that need from 2.5 to 6 M salt (NaCl) in media for optimal growth (Kushner and Kamekura 1988). In contrast to many halophilic organisms, halophilic archaea have the same ionic strength inside the cell as outside. K^+ ions are predominant inside the cell while Na^+ ions are predominant in the growth medium (Madigan and Oren 1999). Therefore all the macromolecules, including the proteins, of the halophilic archaea have to be adapted to function under conditions of high ionic strength (Oren 1999; Ventosa et al. 1998; Dennis and Shimmin 1997; Eisenberg et al. 1992; Lanyi 1974). Most of the studied halophilic proteins function at salt concentrations from 2 to 5 M. Usually they lose their activity and denature at NaCl concentrations of 1–2 M (Madern et al. 2000).

Suggested adaptation mechanisms of halophilic proteins include increasing the content of acidic residues and decreasing that of hydrophobic residues, contributing to the organization of water molecules into an aqueous shell surrounding the protein (Lanyi 1974; Eisenberg 1994).

The application of circular dichroism (CD) and differential scanning calorimetry (DSC) methods is informative in studying changes in protein secondary and tertiary structure, respectively. DSC provides information about thermodynamic parameters of proteins, their stability at different conditions, the domain structure of proteins, their oligomeric state, and the formation of equilibrium intermediates during protein heat denaturation (Privalov 1979; Privalov and Potekhin 1986; Sanchez-Ruiz 1995). In the case of halophilic proteins, DSC was used for studying the stability of aspartate aminotransferase from *Haloferax mediterranei* (Muriana et al. 1991) and flagellins from *Halobacterium halobium* (Tarasov et al. 1995). CD has been used for the registration of structure changes of halophilic malate dehydrogenase during its inactivation (Pundak and Eisenberg 1981; Pundak et al. 1981).

In our previous studies we isolated and characterized the nucleoside diphosphate kinase (NDPK) from the haloalkaliphilic archaeon *Natrialba magadii* (Polosina et al. 1998). The protein is a homooligomeric hexamer with a monomer molecular weight of 23 kDa. The main function of NDPK is to exchange γ-phosphates between nucleoside triphosphates and diphosphates (Uesaka et al. 1987). We showed that *Nm* NDPK is active over a wide range of NaCl, which is not typical of extremely halophilic proteins. The stability of different mesophilic NDPKs was studied by DSC (Giartosio et al. 1996). It was found that nonhalophilic hexameric NDP kinase melts irreversibly because of aggregation. Also it was shown that the addition of adenosine diphosphate (ADP)

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increased its melting temperature. In this work, the kinase stability was studied in more detail by CD, DSC, size-exclusion chromatography, and biochemical methods. A novel possible mechanism of halophile protein stabilization that permits functioning over a wide range of NaCl concentrations is proposed.

Materials and methods

Protein preparation

The *Nm* NDPK was isolated and purified from *N. magadii* [the previous strain name was *Natronobacterium magadii* (Kamekura et al. 1997)] as described previously (Polosina et al. 1998). To obtain a nonphosphorylated protein without ligands, the purified kinase was dialyzed against 20 mM Tris-HCl, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$, containing 5 mM guanosine diphosphate (GDP) and then against this buffer without GDP. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12% acrylamide gels. The protein concentration was measured by Biuret and Lowry procedures using a kit for microdetermination of the total protein (Sigma) with bovine serum albumin as a standard.

Amino acid composition

The protein (0.01–0.05 µmol) was hydrolyzed with concentrated HCl for 24 and 72 h. Determination of amino acid content was carried out on an amino acid analyzer DS-500 (Durrum). The amino acid composition of *Nm* NDPK is given in Table 1.

Activity assay

The kinase activity in different conditions was checked by testing its autophosphorylation according to the method described by Polosina et al. (1998). The kinase was incubated with 30 µCi [γ-^{32}P]ATP for 30 min. To check the kinase activity in the presence of K^+ instead of Na^+, the following buffer was used: 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, containing different concentrations of KCl (3, 2, 1, and 0.5 M). To check the NDPK enzymatic activity at different urea concentrations, the following buffer was used: 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 3.5 M NaCl (and 1.75 M NaCl), containing 0, 1, 2, 3, 4, 5, and 6 (also 7 and 8 for 1.75 M NaCl) M urea. The kinase was incubated in these buffers for 24 h at 4°C. To check recovery of the kinase activity after denaturation, the protein was dialyzed against 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 8 M urea and after denaturation it was dialyzed against 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing different concentrations of NaCl or 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1.75 M NaCl containing different concentrations of urea. To check the kinase activity at different temperatures, the kinase in 20 mM Tris-HCl, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$ was preincubated at 20°, 40°, 60°, 70°, 80°, 85°, 90°, and 95°C for 1 or 10 min before incubation with [γ-^{32}P]ATP. To prevent renaturation, the protein was precipitated with hot 10% tricarboxylic acid (TCA) added immediately after labeling. After incubation, SDS-PAGE sample buffer was added to the reaction mixtures 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. The quantitative changes in NDPK activity were calculated approximately by scanning the radioactive spots.

Table 1. The amino acid composition of nucleoside diphosphate kinase from *Natrialba magadii*

Amino acid	% M	Amino acid	% M
Aspartic acid/asparagine	12.25	Leucine	6.58
Threonine	5.4	Tyrosine	0.97
Serine	3.41	Phenylalanine	3.28
Glutamic acid/glutamine	17.03	Histidine	4.26
Glycine	9.48	Lysine	2.98
Alanine	7.32	Arginine	7.27
Valine	7.47	Proline	4.81
Methionine	2.93	Isoleucine	4.85
Tryptophan	–[a]	Cysteine	ND[b]

[a] Quantity not known. Its presence was determined by fluorescence spectra
[b] ND, not determined

CD and fluorescence measurements

CD spectra were recorded on a JASCO-40 at 20°C in a cuvette of 0.1 cm path-length at 200–260 nm. *Nm* NDPK with a concentration of 1 mg/ml was used in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing NaCl from 90 mM to 1.75 M. For testing at different urea concentrations, NDPK in 20 mM Tris-HCl, 5 mM MgCl, 1.75 M NaCl containing 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.2 M urea; NDPK in 20 mM Tris-HCl, 5 mM MgCl, containing 8 and 10 M urea was used. The fluorescence spectra were obtained with a Hitachi F-3010 fluorescence spectrophotometer with excitation at 295 nm for intrinsic tryptophan fluorescence.

DSC measurements

The calorimetric data were obtained using a SCAL-1 (Scal, Russia) scanning microcalorimeter with a cell volume of 0.33 ml at a heating rate of 1°C/min. To check the dependence of the calorimetric profiles on the heating rate, the latter was changed to 0.25°, 0.5°, or 2°C/min. In order to reach complete pH and ionic equilibration, the protein samples were dialyzed three times against a 100-fold volume buffer excess before the DSC experiments. The DSC data were acquired and analyzed using the

SCAL PC software (Filimonov et al. 1982). The heat capacity of the samples was calculated per mole of monomer, assuming the kinase molecular weight to be 23 kDa. For the calorimetric experiments the following buffers were used: 20 mM HEPES, pH 8.0, 5 mM $MgCl_2$ containing 3.5, 1.75, 0.9 M, and 450 and 180 mM NaCl; 20 mM HEPES, pH 7.0, 3.5 M NaCl, 5 mM $MgCl_2$; 20 mM cacodylate, pH 6.0, 3.5 M NaCl, 5 mM $MgCl_2$; 20 mM veronal, pH 9.0, 3.5 M NaCl, 5 mM $MgCl_2$; 20 mM veronal, pH 10.0, 3.5 M NaCl, 5 mM $MgCl_2$; 20 mM Na_2HPO_4, pH 11.0, 3.5 M NaCl, 5 mM $MgCl_2$. For determining the dependence of *Nm* NDPK stability on ligand presence, 5 mM ATP and 5 mM GDP were used.

Size-exclusion chromatography

NDPK (100 µl with a concentration of 0.5 mg/ml) was applied to a Superose 12 column in a Pharmacia fast protein liquid chromatography (FPLC) system and was eluted with following buffers: 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing 3.5, 1.75 M, 875, 175 mM or 0 M NaCl; 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 8 M urea containing 3.5 or 0 M NaCl; 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.04 mM Tween-20. The flow rate was 0.4 ml/min. The eluted protein was detected by UV absorbance. The column was calibrated in 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0, containing 3.5 M or 175 mM NaCl using the following proteins: β-galactosidase (116 kDa), chymotrypsinogen A (25 kDa), aldolase tetramer (158 kDa), and aldolase dimer (79 kDa).

Results

Maintenance of secondary structure in different conditions

In this study, nucleoside diphosphate kinase from *N. magadii* (*Nm* NDPK) was purified and used for structural investigations. The CD spectra of NDPK were measured in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing NaCl concentrations from 3.5 M to 90 mM. It was shown that essentially the spectra did not change. However at 90 mM NaCl the curve showed a slight decrease in α-helical content indicating the shift to a denaturation state (Fig. 1a).

Temperature dependence of molar ellipticity at 222 nm was measured for NDPK in 20 mM Tris-HCl, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$ containing 5 mM ATP. The secondary structure melting occurred at 70°C and was reversible. The CD spectra measured at 85°C showed preservation of some secondary structure at this temperature (data not shown).

Nm NDPK preserved its secondary structure in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing 1.75 M NaCl and at urea concentrations from zero to 7.2 M. The complete unfolding of the protein was observed in the buffer without NaCl containing 8 or 10 M urea. In Fig. 1b, five of the 18 curves are shown.

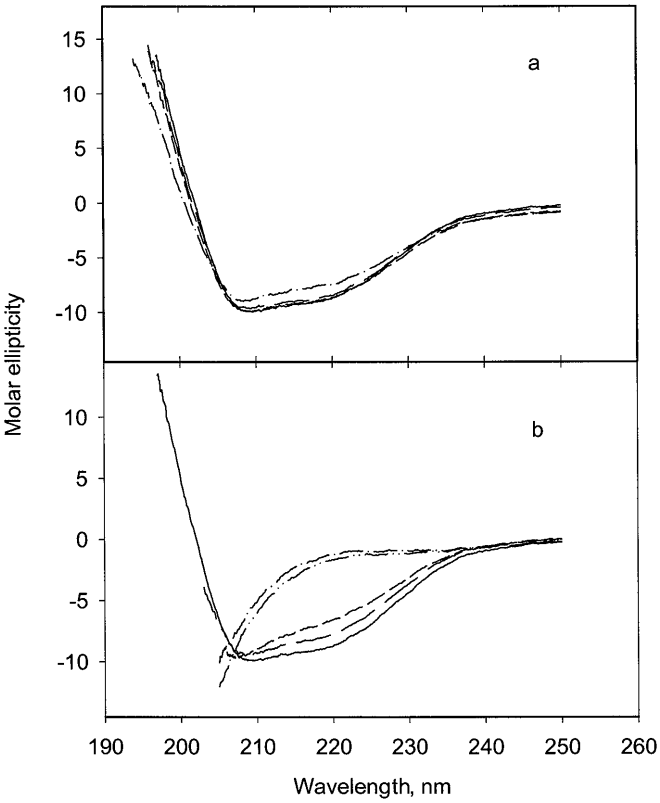

Fig. 1a,b. Far UV circular dichroism (CD) spectra of nucleoside diphosphate kinase from *Natrialba magadii* (*Nm* NDPK) in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ at different NaCl concentrations: **a** 1.75 M NaCl (*solid line*), 0.9 M NaCl (*long-dashed line*), 450 mM NaCl (*dashed line*), zero NaCl (*dash-dotted line*) and different urea concentrations. **b** 1.75 M NaCl, zero urea (*solid line*), 1.75 M NaCl, 3.5 M urea (*long dashed line*), 1.75 M NaCl, 7.2 M urea (*dashed line*), zero NaCl, 8 M urea (*dash-dot-dotted line*), zero NaCl, 10 M urea (*dash-dotted line*)

Maintenance of tertiary structure in different conditions

The DSC experiments of the NDPK in 20 mM Tris-HCl, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$, 5 mM ATP demonstrated that it has a single denaturation transition centered at 70°C, i.e. the same as the secondary structure melting. It was found that the process of melting of the *Nm* NDPK tertiary structure was reversible (Fig. 2). The absence of nucleoside phosphates in the protein solution decreased the *Nm* NDPK melting temperature. At 3.5 M NaCl and pH 8.0 the dephosphorylated kinase melted at 66°C, while in the presence of 5 mM GDP or ATP its melting temperature increased to 68° or 70°C, respectively (Fig. 3). The calorimetric experiments were carried out at the melting rate of 1°C/min. Repetition of these experiments at other melting rates (0.5° and 2°C/min) showed slow kinetics of kinase denaturation, i.e. the denaturation process at such heating rates is nonequilibrium (Fig. 4). At 3.5 M NaCl and pH 8.0 the temperature decreased from 70° to 62°C at the melting rate of 0.5°C/min and to 69°C at 2°C/min.

The DSC experiments showed that the protein is more stable at basic pH. The melting temperature decreased from 73°C at pH 10.0 to 61°C at pH 7.0. A further decrease in pH

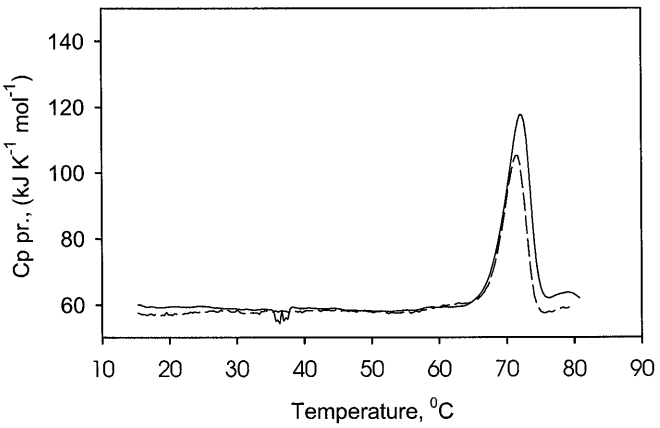

Fig. 2. Temperature dependencies of molar heat capacity for *Nm* NDPK in 20 mM HEPES, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$, 5 mM adenosine triphosphate (ATP). *Solid line* first heating, *dashed line* second heating

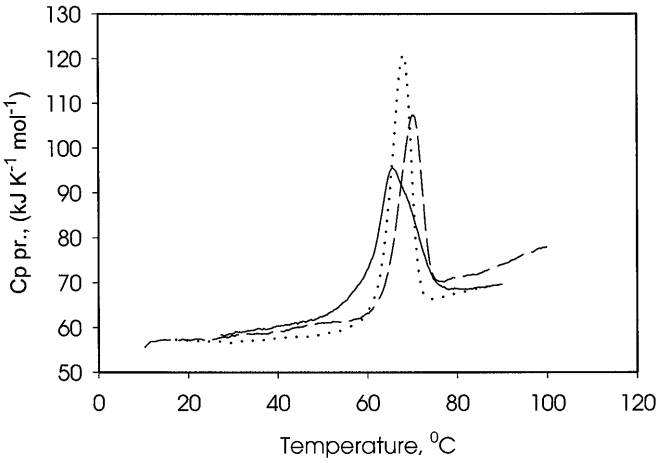

Fig. 3. Temperature dependency of molar heat capacity for *Nm* NDPK in the presence and absence of guanosine diphosphate (GDP) or ATP. The samples (1.6 mg/ml in 20 mM HEPES, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$) were scanned at a heating rate of 1°C/min. Ligand concentration was 0 (*solid line*), 5 mM GDP (*dotted line*), 5 mM ATP (*dashed line*)

destabilized the protein: it rapidly lost its activity and aggregated upon heating at pH 6.0 (data not shown).

According to the DSC analysis, decreasing of the NaCl concentration from 3.5 to 0.9 M led to a decrease in the melting temperature from 70° to 50°C (data not shown).

In the fluorescence experiments with the kinase in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing different NaCl concentrations, the fluorescence maximum shifted from 333 nm at 1.75 M NaCl to 340 nm at zero NaCl. In the buffer with 8 M urea and without NaCl, the fluorescence maximum was at 350 nm, which indicates that the kinase was unfolded.

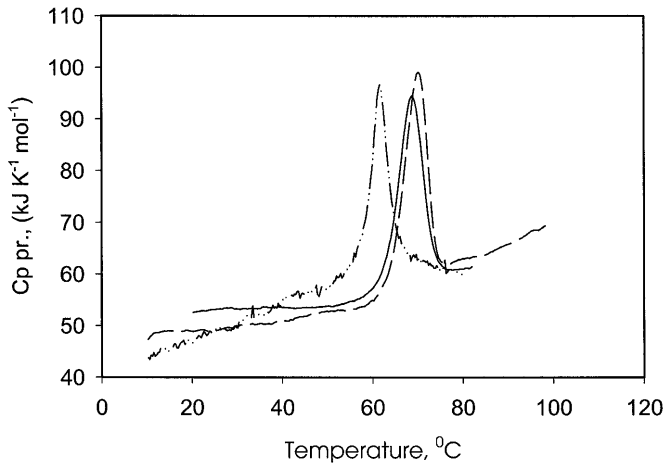

Fig. 4. Temperature dependency of molar heat capacity for *Nm* NDPK in 20 mM HEPES, pH 8.0, 3.5 M NaCl, 5 mM $MgCl_2$, 5 mM ATP. The sample (1.6 mg/ml) was scanned at a heating rate of 0.25°C/min (*dash-dot-dot line*), 1°C/min (*dashed line*), 2°C/min (*solid line*)

Maintenance of quaternary structure in different conditions

The oligomeric parameters of *Nm* NDPK were determined by analytical gel-filtration using protein molecular weight markers.

The gel-filtration experiments showed that the kinase maintained quaternary structure independently of NaCl concentration in the buffers. It had a hexameric form in the buffers containing NaCl from 3.5 to 0 M (Fig. 5a).

In the buffer without NaCl containing 0.04 mM Tween-20 and in the buffer containing 1.75 M NaCl and 8 M urea, the protein also remained in a hexameric form. The kinase dissociation to monomers occurred in the buffer containing 8 M urea and no NaCl (Fig. 5c).

Functional activity of *Nm* NDPK in different conditions

Nm NDPK functions in two steps similar to those in NDP kinases from mesophilic organisms; the first step is the formation of a phosphorylated intermediate (Polosina et al. 1998). To study the NDPK activity in different conditions, its autophosphorylation using [γ-^{32}P]ATP as a substrate was examined.

Previously it has been found that *Nm* NDPK preserves the ability to transfer γ-phosphate from ATP to GDP in the range of NaCl concentrations from 3.5 M to 90 mM (Polosina et al. 1998). It was shown by studying the dependence of *Nm* NDPK activity on the KCl concentration that there is no substantial difference in activity of *Nm* NDPK depending on the type of monovalency cations used (K^+ or Na^+). The protein retained its activity when the concentration of KCl varied from 3 to at least 0.5 M. The data on *Nm* NDPK activity in solutions with different concentrations of NaCl and urea are summarized in Table 2. The activity in the buffer without NaCl and urea was preserved for at least 11 days, though it decreased twice at the end.

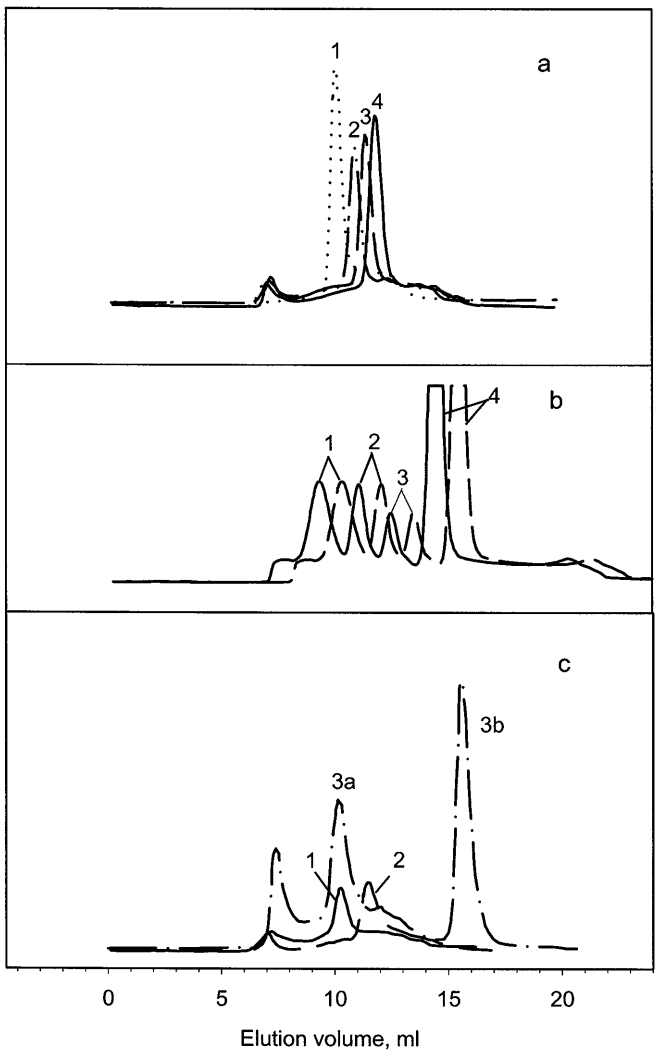

Fig. 5a–c. Analysis of the *Nm* NDPK oligomeric level by size-exclusion chromatography. The protein, preincubated for 24 h in 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing different NaCl and urea concentrations, was injected on a Superose 12 column equilibrated with the same buffer. The proteins used as markers (**b**) were aldolase tetramer (158 kDa, *peaks 1*), β-galactosidase (116 kDa, *peaks 2*), aldolase dimer (79 kDa, *peaks 3*), chymotrypsinogen A (25 kDa, *peaks 4*). Markers were run in the buffer containing 3.5 M NaCl (*dashed line*) and 175 mM NaCl (*solid line*). *Nm* NDPK was preincubated at different NaCl concentrations (**a**): 0 NaCl (*dotted line, peak 1*), 175 mM NaCl (*dash-dotted line, peak 2*), 0.9 M NaCl (*dashed line, peak 3*), 3.5 M NaCl (*solid line, peak 4*). The protein was preincubated in different detergents (**c**): 1.75 M NaCl, 8 M urea (*solid line, peak 1*), zero NaCl, 0.04 mM Tween-20 (*dashed line, peak 2*), zero NaCl, 8 M urea (*dash-dotted line, peaks 3a, b*)

It was shown that *Nm* NDPK in the buffer with 3.5 M NaCl (optimal ionic conditions for *N. magadii*) kept its activity in the presence of high urea concentrations. For example, after 24 h of dialysis against this solution containing 6 M of urea, the protein preserved 30% of its initial activity. Moreover, the kinase kept its activity in the buffer with urea concentrations up to 8 M when NaCl concentration was decreased from 3.5 to 1.75 M, although the activity also decreased. Thus, in the buffer containing

Table 2. Dependence of autophosphorylation activity on NaCl and urea concentration

Urea (M)	0	1	2	3	4	5	6	7	8	9	10	Initial protein condition
NaCl (M)												
0	+	+	+	+	+	–	–	–	–	–	–	N
0	+	–	–	–	–							D
1.75	+	+	+	+	+	+	+	+	+			N
1.75	+	+	+	+	–	–	–	–	–			D
3.5	+	+	+	+	+	+	+					N
3.5	+											D

Presence (+) and absence (–) of activity for protein. (N) Protein was transferred from native conditions (3.5 M NaCl) or (D) denaturing conditions (0 M NaCl, 8 M urea) to experimental conditions by dialysis

1.75 M NaCl and 6 M urea, the kinase activity decreased to 10% of its initial activity. After 4 days incubation at the same conditions, *Nm* NDPK activity had hardly changed. However, decreasing NaCl concentration to zero in the presence of 8 M urea led to rapid deactivation of the protein. The activity of *Nm* NDPK may be completely recovered after dialysis not only against a buffer containing 3.5 M NaCl, but also against a buffer without NaCl. The dialysis against the buffers with different concentrations of urea showed that the activity was restored only in buffers that contained no more than 3 M urea.

Discussion

According to the literature, proteins functioning at high salt concentrations exhibit the following features in comparison with their mesophilic homologues: (1) higher content of acidic residues, (2) lower content of lysine and hydrophobic residues, (3) somewhat higher thermostabilities (Lanyi 1974; Madern et al. 1995).

Although the conventional amino acid analysis does not distinguish between aspartic acid (Asp) and asparagine (Asn) or glutamic acid (Glu) and glutamine (Gln), a comparison of the total amounts of these residues in halophilic and nonhalophilic NDPKs suggests an essential prevalence of the acidic residues in the former. While the total content of these residues in *Nm* NDPK and in *Halobacterium salinarum* NDPK is 29.28 and 24.84 mol%, respectively, their average content in nonhalophilic NDPKs shown in Table 3 is only 18.14 mol%.

At the same time, *Nm* NDPK has only 2.98 mol% of lysine (which is close to 2.48 mol% *H. salinarum*) whereas its average content in the nonhalophilic homologues is much higher (6.28 mol%). A further comparison of the amino acid contents shows that the polypeptide chains of halophilic NDPKs are generally less hydrophobic than those of their nonhalophilic homologues (Table 3). The thermal stability of *Nm* NDPK seems to be higher than that of the mesophilic NDPKs (Giartosio et al. 1996). The active pH range of *Nm* NDPK is wide and similar to that

Table 3. Comparison of the hydrophobicity content in hexameric NDPKs from different organisms

Organism	Reference	Hydrophobic amino acids (% M)	Total hydrophobicity of hydrophobic amino acids
Halophilic			
Natrialba magadii		33.4	28.9
Halobacterium salinarum	Ng et al. (2000)	37.33	31.0
Nonhalophilic			
Human NDPK *A*	Dooley et al. (1994)	38.14	38.4
Human NDPK *D* (mitochondrial)	Tsuiki et al. (1999)	40.9	32.7
Dictyostelium discoideum	Lacombe et al. (1990)	42.09	39.7
Archaeoglobus fulgidus	Klenk et al. (1997)	42.2	35.9
Aeropyrum pernix	Kawarabayasi et al. (1999)	43.64	42.7
Methanococcus jannaschii	Bult et al. (1996)	40.13	40.3
Pyrococcus horikoshii	Kawarabayasi et al. (1998)	43.87	41.9
Bacillus subtilis	Henner et al. (1985)	40.72	42.1
Synechococcus sp.	Kutach et al. (1995)	40.79	38.0
Staphylococcus aureus	Horsburgh et al. (1996)	38.81	38.1

The hydrophobicity was calculated as the sum of tabular values (Nozaki and Tanford 1971) for hydrophobicity of amino acid residues

of malate dehydrogenase from *Haloarcula marismortui* (Hecht and Jaenicke 1989).

One of the most important distinctions between *Nm* NDPK and other halophilic proteins is the higher stability of *Nm* NDPK to urea. While in natural salt conditions *Hm* malate dehydrogenase begins to lose its activity in 2.4 M urea (Hecht and Jaenicke 1989), *Nm* NDPK does not denature completely even after 4 days of incubation in 8 M urea at a NaCl concentration of 1.75 M, which is suboptimal for this protein.

Nm NDPK maintains functional stability in solutions with low ionic strength, which is unusual in the halophilic proteins studied so far. According to the literature, most of the extreme halophilic proteins require a high salt concentration in order to maintain their activity. Some of them dissociate into monomers and denature upon decreasing NaCl concentration to 2–1 M (Madern et al. 2000); on the other hand, *Nm* NDPK remains active even without NaCl.

To explain these properties of *Nm* NDPK, we propose the existence of a specific mechanism that permits the kinase to keep functional activity over a wide range of NaCl concentrations.

It is probable that very strong intersubunit contacts making up the quaternary structure form the basis for higher functional stability. All structures found for mesophilic NDPK are very similar. The subunit core is formed by the single structural domain (Dumas et al. 1992; Biggs et al. 1990; Chiadmi et al. 1993; Postel 1998). However, this does not mean that subunits forming the supramolecular structure are common cooperative subdomains and that they melt as a single entity even in a monomeric form. It is known that globular proteins with a molecular weight exceeding 10 kDa are often composed of some cooperative subdomains. These subdomains can melt cooperatively or individually, depending on the conditions (Llinas and Marqusee 1998). In particular, the formation of a strong supramolecular structure can selectively stabilize and cooperate only those subdomains whose interaction leads to the formation of the quaternary structure.

According to our data, it is possible that *Nm* NDPK monomers may also consist of several subdomains, one or more of which forms intersubunit contacts. These contacts are so advantageous that subdomains stabilized by them cannot be destroyed either by an extreme decrease in the ionic strength or by protein-heating or the influence of urea at a relatively high ionic strength. At the same time, subdomains that are not involved in the formation of intersubunit contacts are probably less stable in the face of a decrease in ionic strength and other destabilizing influences.

The preservation of a large amount of secondary structure at the temperature at which the denaturation transition terminates according to CD and DSC can explain the high stability of the quaternary structure. The absence of the dependence of the protein-melting temperature on its concentration in the DSC experiments demonstrates that the protein does not dissociate into monomers upon melting and, accordingly, the subdomains forming intersubunit contacts do not melt. On the other hand, the specific enthalpy corresponding to the *Nm* NDPK peak heat absorption for most conditions varies from 2.5 to 3.1 cal/g. Such low values of the specific enthalpy show that in this transition only part of the structure melts (Privalov and Khechinashvili 1974). The part of the structure that remains intact is so stable that it cannot be destroyed in the conditions used in the calorimetric experiments. On the whole, these data suggest that only the subdomain not forming intersubunit contacts denatures at thermal denaturation.

The preservation of intersubunit contacts at melting also can explain the *Nm* NDPK renaturation after thermal denaturation, although the studied mesophilic NDPK denatures irreversibly (Giartosio et al. 1996). Furthermore, the irreversible denaturation is typical of most large proteins with quaternary structure as a result of spontaneous aggregation of the unfolded polypeptide chains or

misfolding, especially at the protein concentrations used in calorimetric experiments.

The following facts support this model. In the absence of salt the protein fluorescence spectrum is intermediate between the spectrum of protein in the buffer with 3.5 M NaCl and the spectrum of protein denatured by urea. This indicates that, in the absence of salt, the fluorophore environment is changed and the protein becomes less structured. The assumption about melting of some parts of the protein is also confirmed by the limited proteolysis data published previously (Polosina et al. 1998). The kinase is very stable to protease treatment in solutions with a high salt concentration, but begins to be degraded when transferred to a low salt solution, forming an approximately 14 kDa fragment relatively stable to digestion with trypsin. Probably some parts of the protein denature and become available to protease digestion but stable intersubunit contacts prevent full degradation.

From these data, we conclude that the functional activity of *Nm* NDPK preservation over a wide range of NaCl concentrations is related to the preservation of the quaternary structure.

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