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Oligomeric Structures Determine the Biochemical Characteristics of Human Nucleoside Diphosphate Kinases

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Major human Nucleoside diphosphate kinases (NDPKs) exist as hetero-oligomers, consisting of NDPK-A and NDPK-B, rather than homo-oligomer. To investigate their biological function depending on the oligomeric structure *in vivo*, we characterized the biochemical properties of cellular NDPK. Cellular NDPKs, which are made up of a unique combination of isoforms, were purified from human erythrocyte and placenta. We found that cellular NDPK and recombinant isoforms NDPKs have their own distinct biochemical properties in autophosphorylation, stability toward heat or urea, and DNA binding. Cellular NDPK was found to have unique characteristics rather than the expected additive properties of recombinant isoforms. The mutations in the dimeric interface of NDPK-B (R34G, N69H or K135L) caused defective DNA binding and simultaneously reduced the enzymatic stability. These results suggest that the oligomeric interaction could play a major role in the stability of catalytic domain and might be related to the regulation of various cellular functions of NDPK.

Keywords: Nucleoside diphosphate kinase, Quaternary structure, Heteromeric NDPK, Enzymatic activity, DNA binding property

Introduction

Nucleoside diphosphate kinase (NDPK) is considered to be a housekeeping enzyme for the maintenance of a pool of nucleoside triphosphate that is required for biosynthesis. It catalyzes the transfer of a terminal phosphate group of nucleoside triphosphate to another nucleoside diphosphate. The enzymatic reaction of NDPK involves the formation of a phosphorylated histidine intermediate in the active site. NDPK was known to have broad substrate specificity.

Recently, the interest in NDPK has increased, because several lines of evidence suggest that NDPK has a role in various cellular regulatory processes. NDPK is a homologue of the *Drosophila awd* (abnormal wing disc) gene, and mutations in *awd* are associated with larvae growth and development (Dearolf *et al.*, 1988; Biggs *et al.*, 1990). A modest overexpression of Nm23 inhibited progesterone-induced oocyte maturation by delaying the accumulation of Mos (Kim *et al.*, 2000). Major human NDPKs have been identified as NDPK-A (Nm23-H1) (Rosengard *et al.*, 1989) and NDPK-B (Nm23-H2) (Stahl *et al.*, 1991). NDPK-A acts as a suppressor of the metastasis in some tumor types (Steeg *et al.*, 1988; De La Rosa *et al.*, 1995). Also, NDPK-B is identical to PuF, a protein that binds to a nuclease-sensitive element in the promoter of *c-myc* oncogene, and activates the transcription of the *c-myc* oncogene *in vitro* (Postel *et al.*, 1993; Berberich and Postel, 1995; Postel *et al.*, 1996). This DNA binding activity and transcriptional activation can occur in the absence of enzymatic activity (Berberich and Postel, 1995). The identification of a differentiation inhibitory factor (denoted 'I factor') in cell lysates and conditioned media from mouse myeloid leukemia M1 cells as a member of the NDPK family, suggests a role of NDPK in suppressing differentiation (Okabe-Kado *et al.*, 1992). Recently, new NDPK genes, DR-nm23, nm23-H4 and nm23-H5, were identified (Venturelli *et al.*, 1995; Milon *et al.*, 1997; Munier *et al.*, 1998). DR-nm23 is involved in the control of granulocyte differentiation and in the apoptosis of the myeloid cell (Venturelli *et al.*, 1995). Nm23-H4 contains the domain of NDPK catalysis, nucleotide binding, and an additional NH_2-terminal region that is rich in positively charged residues (Milon *et al.*, 1997). Nm23-H5

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has an additional 55 amino acid COOH-terminus and does not exhibit NDPK enzymatic activity (Munier *et al.*, 1998). Although catalytic NDPKs are multifunctional at the cellular level beyond enzymatic activity, the molecular bases of these functions are poorly understood.

NDPKs are highly conserved throughout the evolution, and the secondary structure is composed of a single domain subunit, the βαββαβ motif. However, the quaternary structure of NDPK varies; the eukaryotic NDPKs are hexamers, while several bacterial NDPKs are tetrameters. Recently, differences between homohexameric NDPK from *D. discoideum* and homotetrameric NDPK from *E. coli* were reported (Giartosio *et al.*, 1996). The report showed that the tetrameric NDP kinase is thermolabile and behaves like the mutant (P105G) of hexameric kinase. Mutation of Pro-100 in the trimeric interface of *Dictyostelium* or *Drosophila* had no effect on the enzymatic activity, but the protein did become unstable, presumably due to the dissociation of the hexamer (Lascu *et al.*, 1992; Karlsson *et al.*, 1996). The quaternary structure affects the stability of the protein, which can be a regulatory mechanism of NDPK in various cellular processes. It appears that cellular human NDPKs have various oligomeric structures; NDPKs from human erythrocytes are heterohexamers (Gilles *et al.*, 1991), while recombinant Nm23-H1 is a homohexamer.

We worked on the hypothesis that the multifunctional nature of NDPK could originate from the existence of various oligomeric structures in cellular and recombinant NDPK. A recent report showed that the oligomeric state is related to the DNA binding properties of NDPK (Mesnildrey, Agou, and Veron, 1997). Here we examined the biochemical properties of various NDPK isoforms that have different oligomeric states (cellular NDPKs from human erythrocytes and placenta, recombinant NDPK-A, and recombinant NDPK-B and its mutants), in terms of enzymatic stability, autophosphorylation and DNA binding property. We found that heterohexameric cellular NDPK from human erythrocytes is the most fragile and NDPK-B the most stable. The stability of NDPK-B was reduced by mutating the dimeric interface (R34G, N69H, K135L). These findings show that the oligomeric interaction of NDPK could regulate the stability of its catalytic domain and its biochemical properties.

Materials and Methods

Protein purification Point substitution mutations in nm23-H2 cDNA (R34G, N69H, and K135L) were introduced as previously described (Postel *et al.*, 1996). Cellular NDPK from human erythrocytes, recombinant NDPK-A and NDPK-B (these plasmids were gifts from Dr. P. S. Steeg at NCI), and mutant NDPK-Bs were purified, as previously described with modified methods (Kim *et al.*, 1997). Briefly, the cellular NDPK was prepared from 2 L of concentrated erythrocytes. The recombinant NDPK was from the *E. coli* strain BL21 (DE3) that was transformed with the expression plasmid for pET3C-nm23, pET3C-nm23-H2, and mutant pET3C-nm23-H2s. Each cytosolic fraction was applied onto a 2-4 ml ATP-sepharose affinity column that was equilibrated with Buffer A (20 mM Tris-acetate, 20 mM NaCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 15 mM β-mercaptoethanol, pH 7.4) at a flow rate of 0.5 ml/min. The column was washed with Buffer A containing 0.25 M NaCl, and eluted with Buffer A containing 1 mM ATP or 1 mM GTP.

NDPK activity assay The NDPK enzymatic activity was measured with the modified method, as described previously (Haut *et al.*, 1991; Hailat *et al.*, 1991). Enzymes were incubated with a reaction buffer (10 μl) containing 20 mM HEPES, pH 7.4, 0.1 mM each of ATP and UDP as substrate, 0.5 μCi [γ-^{32}P]ATP, and 3 mM $MgCl_2$ at 37°C for 10 min. The reaction was stopped by adding the gel sample buffer that contained 125 mM Tris base, 2.3% SDS, 10% glycerol, and 5% β-mercaptoethanol. Aliquots were applied to the polyethyleneimine (PEI) cellulose TLC plate and developed in a solution of 0.75 M KH_2PO_4 (pH 3.6). An autoradiogram was obtained by exposing the dried TLC plate to X-ray film. The formation of [γ-^{32}P]NTP was quantified using a Packard Instant Imager (Mariden, CT, USA).

***In vitro* NDPK autophosphorylation** The purified NDPKs (50-500 ng) from human erythrocytes and placenta, recombinant NDPK-A and NDPK-B, and mutant NDPK-Bs were autophosphorylated with 0.5 μCi [g-^{32}P]ATP in 10 μl of a reaction buffer (20 mM HEPES, 1 mM ATP, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 3% glycerol, pH 7.9) at room temperature for 20 min. The autophosphorylated proteins were analyzed on 5% vertical non-denaturing polyacrylamide gel, 10% SDS-PAGE according to Laemmli (Laemmli, 1979), or by a two-dimensional gel electrophoresis. For the first dimensional isoelectrofocusing, the electrode was reversed to resolve the basic NDPK-B. The protein bands or spots were dried and detected with BAS 2500 (Fujiphoto Film, Tokyo, Japan).

Capillary electrophoresis Capillary electrophoresis was carried out, as described previously for the NDPK analysis based on pIs (Heo *et al.*, 1997). An untreated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA), 40 cm long (31.5 cm to detector × 50 μm i.d.), was used as a separation column. An automated HP^{3D}CE system (Hewlett Packard, Waldbronn, Germany) with HP^{3D}CE Chemstation for a control and data acquisition was used for this work. HP^{3D}CE was equipped with a photodiode array UV detector, an automatic pressure or an electrokinetic sample injector, autosampler, a peltier temperature controller, and a 30 kV power supply. Prior to each run, the capillary was rinsed with 0.1 M NaOH, distilled water, and the running buffer. The capillary was filled with a running buffer, 20 mM sodium borate buffer at pH 9.6, as reported previously (Lee and Heo, 1991). The samples were introduced by a hydrodynamic injection (4×10^3 Pa for 10s), run at a voltage of 20 kV at 25°C, and each protein peak detected at 200 nm.

Immunoprecipitation of heteromeric NDPK The association between NDPK isoforms was examined by immunoprecipitating NDPK that was purified from human placenta with a specific anti-NDPK-A monoclonal antibody (provided by Dr. N. Kimura at Tokyo Metropolitan Institute). Immunoprecipitations were performed by incubating equivalent amounts of purified placenta

NDPK and the antibody in a buffer that contained 2.5 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 15 mM KCl, 0.005% NP40, and 1% glycerol for 1 h at 4°C. An immune complex was mixed with Sepharose-protein A beads for 3 h at 4°C, precipitated by centrifugation, and washed three times with PBS. The reaction was stopped by adding the gel sample buffer. The samples were then subjected to 13% SDS-PAGE and detected by Western analysis using a polyclonal antibody against NDPK.

DNA mobility shift assay The DNA binding property of NDPK isoforms were assessed using a modified electrophoretic mobility shift assay (Postel *et al.*, 1993; Postel and Ferrone, 1994). A typical reaction was carried out in 10 μl of a binding buffer (25 mM HEPES, 0.5 mM EDTA, 0.5 mM DTT, 50 mM KCl, 1% NP-40, and 5% glycerol, pH 7.9) that contained 1-2 ng of an end labeled DNA probe of 102 bp of c-*myc* promoter region (–97~–198), 10-1000 ng of purified NDPK from human erythrocytes, recombinant NDPK-A or NDPK-B, and 50 ng of poly(dI-dC) as a non-specific competitor. All of the binding mixtures were incubated at 4°C for 20 min, mixed with 1 ml of a loading dye (12.5 mM HEPES, 0.25 mM EDTA, 0.25 mM DTT, 25 mM KCl, 0.5% NP40, 2.5% glycerol, and 0.01% xylene cyanol-bromophenol blue, pH 7.9), and separated on a 5% non-denaturing polyacrylamide gel (29% acrylamide and 1% bisacrylamide, w/v) in 0.4 × TBE buffer. Following electrophoresis in a horizontal gel assembly for 2 h at 100 V, the gels were dried and exposed on XRP film (Kodak), or the bands in the gel were quantified with a Packard Instant Imager.

Results

Cellular forms of major human NDPKs consist of NDPK-A and NDPK-B. To determine the characteristics of a cellular NDPK, we first purified the cellular protein from human erythrocytes and placenta, and the recombinant isoforms from *E. coli* expressing nm23-H1 and nm23-H2 by one-step ATP-sepharose affinity column chromatography to a greater than 95% homogeneity. Then the biochemical characteristics of the purified cellular NDPK were compared with those of recombinant NDPK-A and NDPK-B.

Oligomeric structure of human cellular NDPK NDPKs, purified from human erythrocytes and placenta, were found to exist in the form of heteromeric combinations of NDPK-A and NDPK-B, rather than the simple mixtures of NDPK-A and NDPK-B. Purified NDPK from human erythrocytes was made up of NDPK-A and NDPK-B in the ratio 1 : 1, and that from the human placenta in the ratio 1 : 3, as judged by the reducing SDS-PAGE (Fig. 1A). The oligomeric structures were monitored using a non-denaturing gel (Fig. 1B) and capillary electrophoresis (Fig. 1D), in which the proteins were separated according to isoelectric points. The calculated pIs of NDPK-A and NDPK-B are 6.13 and 8.64, respectively. Each recombinant NDPK-A and NDPK-B showed a homogeneous band (Fig. 1B). As expected, the basic NDPK-B moved slowly toward the anode in the native gel electrophoresis and migrated faster toward the cathode than NDPK-A in the

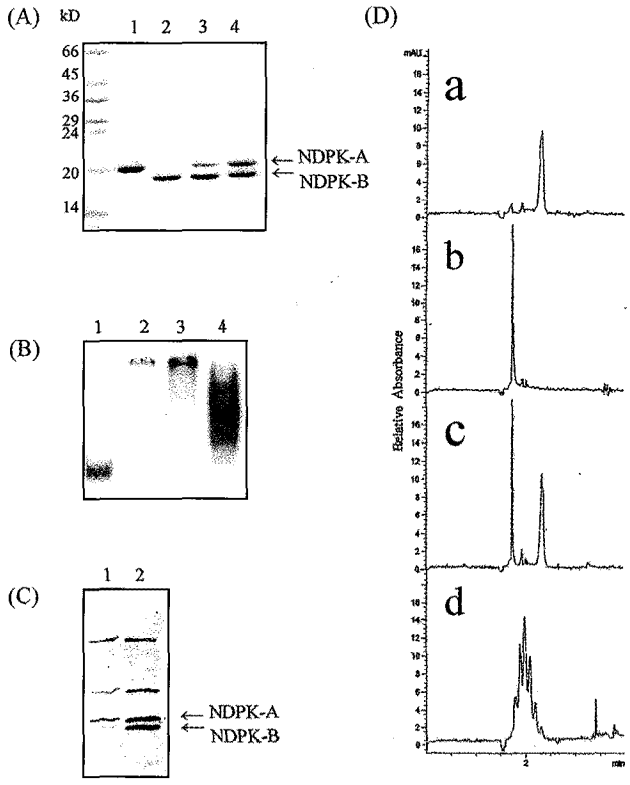

Fig. 1. Separation of purified NDPKs on SDS-PAGE (A), non-denaturing PAGE (B) and capillary electrophoresis (D). (A) Purified recombinant NDPK-A (lane 1), NDPK-B (lane 2), cellular NDPK from human placenta (lane 3) and from human erythrocytes (lane 4) were separated on 12% SDS-PAGE and stained with Coomassie blue. (B) NDPKs autophosphorylated with 0.5 μCi [γ-^{32}P]ATP (lanes are the same as in (A)) were separated on 5% non-denaturing PAGE and subjected to autoradiography. (C) Immunoprecipitated proteins of a mixture of NDPK-A and NDPK-B (lane 1) or human placenta NDPK (lane 2) by an anti-NDPK-A antibody, were separated on SDS-PAGE and detected by Western blot analysis using a polyclonal antibody against NDPK. (D) Recombinant NDPK-A (a), NDPK-B (b), a mixture of recombinant NDPK-A and NDPK-B (c), and cellular NDPK from human erythrocytes (d) were separated by capillary electrophoresis in a 40-cm (31.6 cm to UV detector) × 50 mm I.D. bared fused silica column. CE conditions were as follows: a running buffer, 20 mM sodium borate buffer (pH 9.6); applied voltage, 20 kV; temperature, 25°C; injection, by pressure at 4×10^3 Pa for 10 s; detection wavelength, 200 nm.

capillary electrophoresis (Fig. 1D). Cellular NDPK from erythrocytes migrated between recombinant NDPK-A and NDPK-B. They exhibited five bands that were identified on the gel during non-denaturing gel electrophoresis, which corresponded well with the separation profile that was obtained by capillary electrophoresis (Fig. 1D-d). This indicates that the cellular NDPK from human erythrocytes has heterogeneous populations, which are possibly heterohexamers of NDPK-A and NDPK-B with different pIs (AB_5, A_2B_4, A_3B_3, A_4B_2, A_5B). A_3B_3 was identified as the main

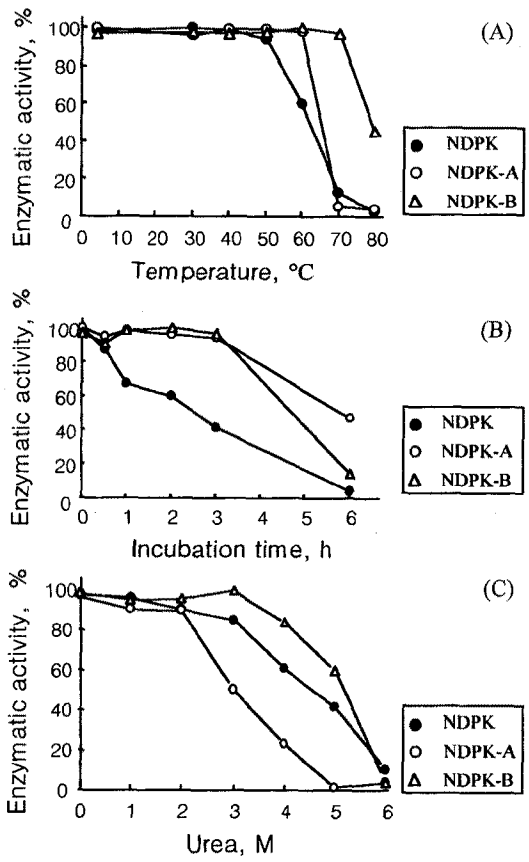

Fig. 2. Thermal (A, B) and urea (C) effects on the enzymatic stability of NDPKs: recombinant NDPK-A (○), NDPK-B (△) and cellular NDPK from erythrocyte (●). Proteins incubated at various temperatures for 10 min (A) or at 37°C for various lengths of time (B), and equilibrated with various concentrations of urea at room temperature for 1 h (C), were assayed as described in "Material and Methods".

peak, while A_6 and B_6 were present in almost negligible amounts (Fig. 1D-d). This agrees with the previous report in which the heterohexameric NDPKs from human erythrocytes were shown (Gilles *et al.*, 1991).

Cellular NDPK from human placenta also showed several populations in the non-denaturing gel. However, the main band occurred in the same position as NDPK-B and extra minor heteromers appeared (Fig. 1B, lane 3). To test whether placenta NDPK that consists of two populations (for example, free NDPK-B and heteromeric NDPK composed of NDPK-A and NDPK-B), or NDPK-A in placenta is associated with NDPK-Bs, we immunoprecipitated placenta NDPK using a specific anti-NDPK-A antibody. When a mixture of recombinant NDPK-A and NDPK-B, which are not associated with each other under normal conditions, was immunoprecipitated with the anti-NDPK-A antibody, only NDPK-A was detected (Fig. 1C, lane 1). On the other hand, immunoprecipitates of placenta NDPK with the anti-NDPK-A antibody were composed of 1 : 1 mixture of NDPK-A to NDPK-B (Fig. 1C, lane 2). This supports the theory that placenta NDPK consists of pure NDPK-B and heteromeric 1 : 1 mixture of NDPK-A and NDPK-B. When recombinant NDPK-A and NDPK-B were mixed in the non-denaturing condition, no interaction was found (Fig. 1D-c.) Therefore, recombinant NDPK-A and NDPK-B, cellular NDPK from human erythrocytes and placenta are composed of the same subunits, but have different oligomeric structures. The oligomeric states of various NDPK proteins were determined by size exclusion chromatography on FPLC or light scattering analysis. Recombinant NDPK-A and cellular NDPK from erythrocytes were hexameric, while recombinant NDPK-B was a mixture of 6-mer, 12-mer, and 24-mer in the presence of 1 mM ATP (data not shown).

Enzymatic stability of NDPKs We tested the stability of proteins to see if various NDPKs have different biochemical characteristics, depending on their oligomeric structures. Each purified NDPK was exposed to heat and urea. The thermostability of NDPK was investigated by measuring the residual enzymatic activity of proteins that were incubated at various temperatures for 10 min (Fig. 2A), or incubated at 37°C for various lengths of time (Fig. 2B). Recombinant NDPK-B was found to have 50% residual activity up to 80°C, NDPK-A up to 65°C, and cellular NDPK from human erythrocytes up to 60°C, respectively. Simultaneously, the activity of NDPK rapidly decreased upon incubation at 37°C for less than 3 h, while recombinant NDPK-A and NDPK-B retained almost 100% activity (Fig. 2B).

The susceptibility of NDPK proteins toward urea denaturation was examined by incubating each NDPK with various concentrations of urea for 1 h at room temperature (Fig. 2C). Urea rapidly inactivated recombinant NDPK-A and NDPK-B at 2 and 5 M, respectively, while cellular NDPK from human erythrocytes gradually lost activity over the range 2-6 M. As was the situation in the case of thermal denaturation, recombinant NDPK-B was most stable in the denaturation with up to 5 M urea.

Autophosphorylation stability of NDPKs Since autophosphorylations of NDPK at His118, Ser44, and Ser120 residues were important in NDP kinase activity and other biological functions (MacDonald *et al.*, 1993; Postel and Ferrone, 1994), thermostability in both enzymatic activity and autophosphorylation of various NDPK proteins were investigated (Fig. 3). Each NDPK protein was heated for 10 min at various temperatures and then divided into two fractions. One fraction was autophosphorylated by labeling the proteins with [γ-^{32}P]ATP for 20 min at room temperature and separated on one-dimensional SDS-PAGE (Fig. 3A). The other fraction was used for enzymatic assay by incubating the proteins with [γ-^{32}P] ATP and UDP as substrates, then separating the reaction mixture on a PEI-cellulose TLC plate (Fig. 3B). The thermostability for enzymatic activity proved similar to that for autophophorylation: NDPK from human

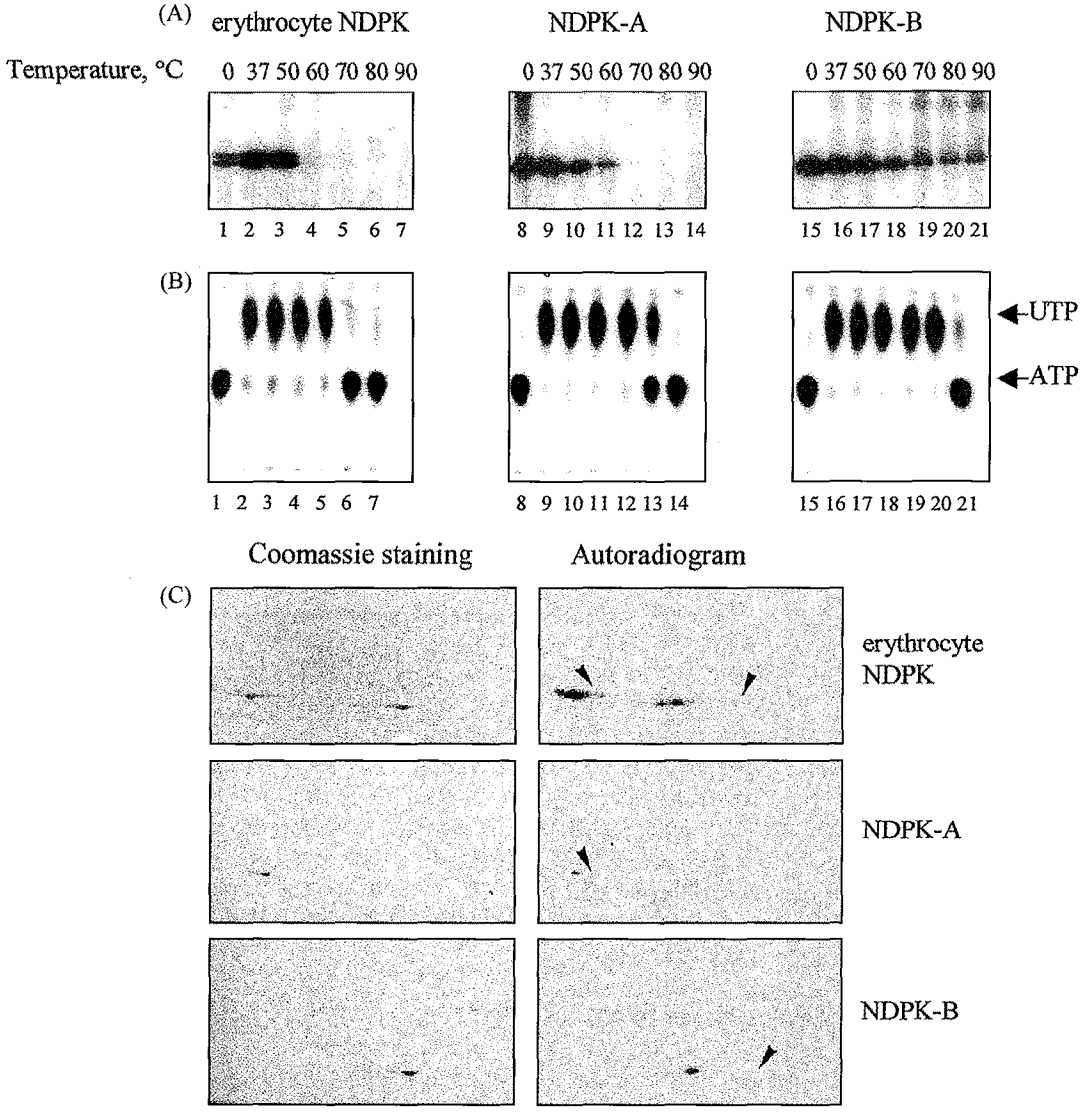

Fig. 3. Autophosphorylation profile of NDPKs toward thermal denaturation. Purified NDPKs were incubated at various temperatures for 10 min and each sample was divided into two, one for autophosphorylation by incubation with [γ-^{32}P]ATP (A) and the other for an enzymatic activity assay (B). Lanes 1-7, cellular NDPK from erythrocyte; lanes 8-14, NDPK-A; lanes 15-21, NDPK-B. Autophosphorylated NDPKs (C), erythrocyte NDPK (a), NDPK-A (b), NDPK-B (c) were separated on a 2-D gel electrophoresis, stained with Coomassie blue, and detected with autoradiography. Arrowheads in the autoradiogram indicate the Coomassie blue staining spots.

erythrocytes was thermally the least stable and recombinant NDPK-B the most stable. But, there were significant discrepancies between enzymatic activity and autophosphorylation at temperatures higher than 50°C and at 0°C respectively. The thermal stability for enzymatic activity was higher than that for autophosphorylation in NDPK-A and cellular NDPK from erythrocytes, but NDPK-B had little enzymatic activity at 90°C despite its continued autophosphorylation. At 0°C, all of the isoforms had no enzymatic activity, even though autophosphorylation occurred. The discrepancy between autophosphorylation and enzymatic activity are thought to be caused by the fact that phosphorylated intermediates are formed during the enzymatic reaction, but the stability of these intermediates would vary depending on the degree of denaturation and the isoforms of NDPK.

To investigate the profile of the phosphorylated intermediates, cellular NDPK and recombinant isoforms were autophosphorylated with [γ-^{32}P]ATP for 20 min at room temperature and separated on two-dimensional SDS-PAGE (Fig. 3C). Recombinant NDPK-A and NDPK-B showed one distinct phosphorylation spot, however, cellular NDPK from human erythrocytes exhibited multiple spots of both NDPK-A and NDPK-B. Thus, several phosphorylation forms could exist only for the cellular NDPK from erythrocytes. Several studies demonstrated that, in addition to the phosphohistidine

intermediate, NDPK can autophosphorylate serine residues (Ser44 and Ser120) (MacDonald *et al.*, 1993; Postel and Ferrone, 1994; Bominaar *et al.*, 1994). This suggests that heterogeneous autophosphorylation can occur in heteromeric NDPK from human erythrocytes. This indicates that autophosphorylation patterns vary depending on the oligomeric combinations of NDPK.

The phosphorylated fraction of total NDPK *in vitro* was less than 2%. In the results shown in Fig. 3, the main protein spot stained with Coomassie blue was not phosphorylated, and the radiolabeled phosphorylation spots were not detected by Coomassie staining (Fig. 3C). It would be expected that the phosphorylation fraction could be increased by cellular protein kinase *in vivo* (data not shown). This suggests that heteromeric cellular NDPK could have various phosphorylation states *in vivo*.

DNA binding properties of NDPKs NDPK-B (Nm23-H2) was identified as a DNA-binding protein and transcriptional activator of the human *c-myc* gene, known previously as PuF (Postel *et al.*, 1993; Berberich and Postel, 1995; Ji, Arcinas and Boxer, 1995; Postel *et al.*, 1996). To determine whether the oligomeric structure of NDPK might affect the DNA binding properties, we examined the DNA binding properties of such NDPK proteins as cellular NDPK from human erythrocytes, recombinant NDPK-A, and NDPK-B by performing an electrophoretic mobility shift assay with double-stranded oligonucleotide that represent the *c-myc* NHE element as a target sequence. Surprisingly, the cellular NDPK from human erythrocyte, as well as recombinant NDPK-B, was found to have DNA binding activity, but there was a difference (Fig. 4A). Cellular NDPK seems to interact with DNA with weak bonding, compared with the NDPK-B • DNA complex.

Despite the amino acid homology between NDPK-A and NDPK-B, their DNA binding modes seem to be completely different, which suggests that unconserved amino acid residues of these two isoforms are responsible for the disparity. With this premise in mind, the amount of DNA binding of cellular NDPK should be half that of recombinant NDPK-B, because native NDPK from human erythrocytes is composed of 1 : 1 mixture of NDPK-A and NDPK-B. However, the DNA retardation pattern of cellular NDPK was completely different from that of recombinant NDPK-B, which suggests that the oligomeric structure of each isoform may be a critical factor in DNA binding. To determine whether the oligomeric structural changes of NDPK-B affect the DNA binding, the effect of thermal denaturation on DNA binding was investigated in 1 : 1 mixture of NDPK-A and NDPK-B, and NDPK-B itself. As in Fig. 4B, the mixture of recombinant NDPK-A and NDPK-B showed DNA binding activity similar to the recombinant NDPK-B. However, DNA binding ability completely disappeared after heating the mixture above 50°C (Fig. 4B, lanes 4-6), which is in contrast to that of recombinant NDPK-B itself (Fig. 4B lanes 9-11).

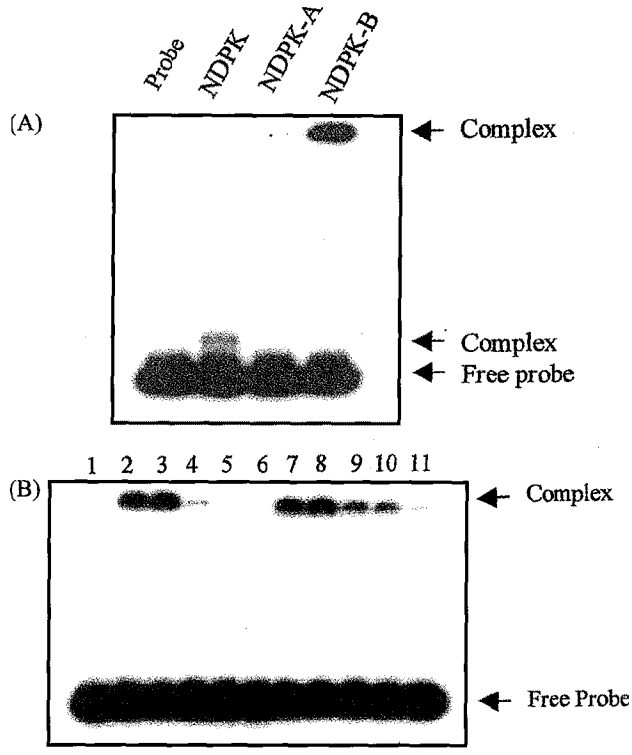

Fig. 4. Electrophoretic mobility shift assay showing DNA binding activity of NDPKs. (A) A radiolabeled probe (1.5 ng) was incubated with 500 ng of each purified protein: lane 1, control probe; lane 2, erythrocyte NDPK; lane 3, NDPK-A; lane 4, NDPK-B. (B) A radiolabeled probe (lane 1) was incubated with a mixture of recombinant NDPK-A and NDPK-B (lanes 2-6), and NDPK-B (lanes 7-11) at various temperatures: lanes 2 and 7, 4°C; lanes 3 and 8, 30°C; lanes 4 and 9; 50°C; lanes 5 and 10, 70°C; lanes 6 and 11, 98°C

NDPK-A and NDPK-B did not interact below 50°C. However, the association between the two proteins was reported previously by Heo *et al.* (1997) that the DNA binding ability was lost with the association of NDPK-B with NDPK-A. These findings indicate that DNA binding residues come from the unconserved amino acids between NDPK-A and NDPK-B, as suggested previously (Postel *et al.*, 1996). Also, that the correct oligomeric structure appears to be required for the correct function of NDPK-B as a transcription factor.

Biochemical properties of mutant NDPK-Bs NDPK-B was very stable for enzymatic activity, autophosphorylation, and DNA binding property. To determine the origin of NDPK-B stability, we examined the properties of the dimeric interface mutants of NDPK-B, which are critical for DNA binding as identified by Postel *et al.* (Postel *et al.*, 1996; Postel and Ferrone, 1994). Mutations of NDPK-B (R34G, N69H and K135L) affected their DNA binding activity (Fig. 5A). K135L was the most critical mutant protein for DNA binding, even these mutants are hexameric and have normal NDP kinase activity. Mutants at histidine 118 lost their enzymatic activity,

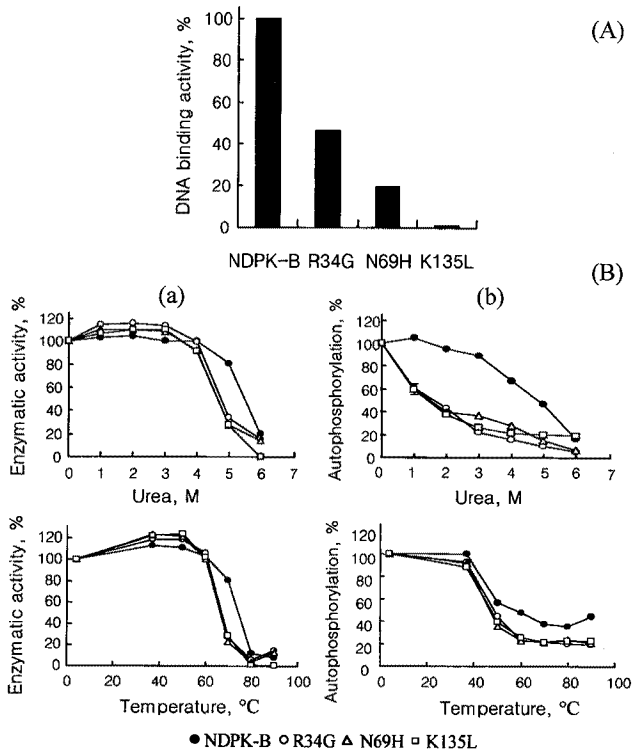

Fig. 5. DNA binding activity (A) and protein stability (B) of mutant NDPK-Bs. (A) The same mobility shift assay as Fig. 4 was used for the wild type NDPK-B and mutant NDPK-Bs, R34G, N69H and K135L. (B) Stability of mutant NDPK-Bs, R34G (○), N69H (△), and K135L (□), toward heat and urea was compared with wild-type NDPK-B (●), in terms of enzymatic activity (a) and autophosphorylation (b). 500 ng of mutant NDPK-Bs were incubated at various temperatures for 10 min or treated with various concentrations of urea at room temperature for 1 h.

the DNA binding activity. This confirms an earlier conclusion that was drawn from the properties of the active site His118 mutant that NDPK has two separate functional domains (i.e. a catalytic domain and a DNA-binding domain.)

To determine whether the catalytic domain is completely isolated from the DNA binding domain, we examined the stability of the catalytic domain in mutants that lack DNA binding ability. As in Fig. 5B, mutants R34G, N69H, and K135L showed similar enzymatic activity and autophophorylation characteristics as the wild-type, though the DNA binding characteristics varied. However, when the stabilities of the NDPK-B mutants were compared with those of the wild-type NDPK-B in terms of NDPK catalytic activity and autophosphorylation, then all three of the mutants were much less stable than the wild-type. These results suggest that modification of the DNA binding domain at the dimeric interface could affect the stability of the catalytic domain.

Discussion

We confirmed that major human cellular NDPKs *in vivo* exist as various heteromers that consist of closely related isoforms, NDPK-A and NDPK-B. In addition, the cellular NDPK was demonstrated to have unique biochemical properties, rather than the additive ones of NDPK-A and NDPK-B. Based on these results, we conclude that the oligomeric interaction between isoforms plays an important role in determining the protein stability for its catalytic and DNA binding properties.

Various human NDPK proteins (cellular NDPKs from human erythrocytes and placenta, recombinant NDPK-A and NDPK-B, and mutant NDPK-B (R34G, N69H and K135L)) were purified to apparent homogeneity using a one-step ATP-sepharose affinity column chromatography, as described previously (Kim *et al.*, 1997). These purified NDPK proteins had a level of stability sufficient for these studies, because nucleotide substrates, such as ATP and GTP, were present in the process of protein purification and storage buffer. Recently Veron *et al.* demonstrated that the dimeric *Dictyostelium* P100S-N150stop NDPK could be re-associated into the hexameric active enzyme in the presence of ATP (Mesnildrey *et al.*, 1998). This is one of evidences that the binding of nucleotides into the catalytic site affects oligomeric association.

Human major NDPKs are composed of NDPK-A and NDPK-B in 1 : 1 ratio and they have various oligomeric populations with their isoforms. In these studies, the human erythrocyte NDPK was confirmed to have various hexameric populations (AB_5, A_2B_4, A_3B_3, A_4B_2, A_5B) by native gel electrophoresis and capillary electrophoresis. A_3B_3 is the main component of the population, while the homohexameric populations of A_6 and B_6 are almost negligible contributors in their native condition, which was in agreement with previous results (Gilles *et al.*, 1991). While the human placental NDPK is composed of NDPK-A and NDPK-B in 1 : 3 ratio (Fig. 1A), it turned out that NDPK-A is associated with NDPK-B in a ratio of nearly 1 : 1, and that the extra NDPK-B is present as a homomer in the placenta. An explanation is that the heteromeric hexamers of 1 : 1 mixture of NDPK-A and NDPK-B exist in cytosol, and that additional homomers of NDPK-B might be in the nucleus, as described previously (Kraeft *et al.*, 1996). Additionally, the amount of NDPK-B in all of the rat tissues and tissue-cultured cells was 2-5 times higher than that of NDPK-A, and NDPK-B was an enriched nuclear fraction (data not shown). This also supports the hypothesis that NDPK-A may associate with NDPK-B in cell cytosol and in nucleus-free erythrocyte, whereas NDPK-B homomers are in nucleus.

The biochemical properties of cellular NDPK from human erythrocytes were compared with those of recombinant NDPK-A and NDPK-B to determine whether it has the additive properties of NDPK-A and NDPK-B. Cellular erythrocyte NDPK was hexameric, and its enzymatic activity and autophosphorylation was comparatively unstable when treated with heat or urea. On the contrary, NDPK-B was very stable and consisted of a mixture of multimeric form, 12 mer and 24 mer as well as 6 mer, in the presence of ATP (data not

shown). These results suggest that the biochemical properties of cellular NDPK may be determined by the oligomeric structure, as well as the composition of subunits.

The distinct differences of autophosphorylation among the various NDPK were observed along with protein stability and phosphorylation patterns. Autophosphorylation of NDPK *in vitro* yielded not only the acid-labile phosphohistidine intermediate activity, but also the acid-stable phosphorylated form at serine residue. This serine phosphorylation of Nm23 correlates with the suppression of tumor metastatic potential in the TK melanoma model system (MacDonald *et al.*, 1993; Postel and Ferrone, 1994). Even though the functions of these phosphorylations are unclear, it is known that histidine phosphorylation is required for serine phosphorylation. The phosphorylated fraction of NDPK *in vitro* accounted for less than 2%. In this present work, heteromeric erythrocyte NDPK showed several phosphorylated spots in 2D-PAGE, while recombinant NDPK-A and NDPK-B exhibited a single phosphorylated spot. Also, there were significant time course discrepancies between enzymatic activity and autophosphorylation at the temperatures higher than 50°C and 0°C respectively. The thermal stability of enzymatic activity was higher than that of autophosphorylation in NDPK-A and cellular NDPK from erythrocytes, but NDPK-B was found to have an absolutely reduced enzymatic activity over 80°C, despite its continued ability to autophosphorylate. This discrepancy can be hypothesized by suggesting that the phosphorylated intermediates were formed during the enzymatic reaction, but that the stability of these intermediates varies and depends on the degree of denaturation and the type of isoforms. More than a ten-fold increase in autophosphorylated NDPK production was observed in the presence of specific proteins in the membrane fraction (unpublished data). Thus, the modulation of autophosphorylation could occur via various cellular mechanisms. This hypothesis awaits further investigation.

The DNA binding activity of NDPK-B was recently reported to bind preferentially single-stranded pyrimidine-rich sequences (Hildebrandt *et al.*, 1995; Agou *et al.*, 1999). In this study, the DNA binding properties of various NDPKs (including NDPK from human erythrocytes, recombinant NDPK-A, and NDPK-B) were characterized using electrophoretic a mobility shift analysis (EMSA). We found that the NDPK-B • DNA complex made a supershifted band. Cellular erythrocyte NDPK also has DNA binding activity, but exhibits a smaller shift than does the NDPK-B • DNA complex. The supershift of NDPK could be due to the properties of NDPK, when it was purified by ATP affinity column, forming a mixture of 12-mer and 24-mer, as well as a hexamer and a very basic protein. On the other hand, erythrocyte NDPK is a hexameric and neutral protein, so the binding of the labeled DNA probe shows only a small change in mobility. This suggests that the oligomeric structure may be a critical factor in DNA binding. A mixture of recombinant NDPK-A and NDPK-B (1 : 1) shows similar DNA binding property as NDPK-B. However, this DNA binding ability completely disappeared by the association of NDPK-A and NDPK-B above 50°C, as described previously (Heo *et al.*, 1997). This suggests that the DNA binding property of NDPK originate from NDPK-B and quaternary structural changes by association with NDPK-A declined this binding. It was previously reported that the amino acid residues (Arg-34, Asn-69 and Lys-135) are critical for DNA binding (Postel *et al.*, 1996). These residues reside on the surface in the proximity of the two-fold axis of adjacent sub-units in the oligomeric status.

It was reported that NDPK-B mutants (R34G, N69H, K135L) lose their DNA binding properties, even if they maintain the same catalytic activity (Postel *et al.*, 1996). In this work, we found that the three mutants have different properties with respect to DNA binding, but that their enzymatic activity, and autophosphorylation pattern to urea and thermal denaturation, are very similar. Three mutants have similar enzymatic and autophosphorylation properties as the wild NDPK-B, but their stabilities are very distinct from that of the wild type. For example, they are more sensitive to denaturation than the wild type. This can be explained on the basis that the mutation of the dimeric interface can affect the stability of the catalytic domain. These results correspond well with the fact that the addition of nucleotide to the active site converts the dimeric *Dictyostelium* P100S-N150 stop mutant to the hexamer (Mensnildrey *et al.*, 1998). The modification of the oligomeric binding site in the molecular surface affects the stability of the inner domain catalytic sites and vice versa. Recently we proposed that the intermolecular crosslinking of Cys109-Cys109 in the oligomeric binding site reduced the enzymatic activity by changing the oligomeric state from hexamer into dimer (Song *et al.*, 2000).

In summary, we purified the various NDPKs and identified the existence of the various oligomeric populations of NDPKs. Changes in the oligomeric structure determine the characteristics and stability of catalytic activity, autophosphorylation, and DNA binding properties. The existence of various oligomeric populations in cellular NDPK can be one way to fine-tune the NDPK functions, if necessary. This regulatory mechanism of multifunctional NDPK should be further investigated.

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