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Recombinant rat nucleoside diphosphate kinase isoforms (α and β): purification, properties and application to immunological detection of native isoforms in rat tissues

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Abstract

We previously demonstrated that at least two isoforms of nucleoside diphosphate (NDP) kinase, the products of two different tandemly arrayed genes, are present in rat. To understand the physiological role of each isoform, some biochemical properties of recombinant rat NDP kinase α- and β-isoforms, produced in large amount, were studied. cDNAs of the two isoforms were inserted in an expression vector pET3b and recombinant enzymes were overproduced in *Escherichia coli*. Their primary structures were different from the native enzymes in that the latter suffer from modification of the NH_2-terminal end. The two recombinant isoforms were purified from the cell lysate to apparent homogeneity by ammonium sulfate fractionation followed by three successive column chromatographies. Despite their extreme similarity in the amino-acid sequences, the two showed somewhat different enzymic properties in terms of di- and triphosphate nucleotide substrate specificity. They showed similar mobilities on SDS-PAGE as expected from their calculated molecular weight (α-isoform, 17283 versus β-isoform, 17192) but differed in isoelectric point (α-isoform, p*I* 6.7; β-isoform, p*I* 7.8) and heat stability. Polyclonal antibody which reacted with both isoforms and α-isoform-specific monoclonal antibodies differentially recognized native enzymes from rat tissues after the tissue extracts were separated by isoelectric focusing gel electrophoresis under a denaturation condition. The results showed that the α-isoform, though its amount varied from one tissue to another, was the major form in rat tissues examined compared with the β-isoform which was detectable in brain and testis. There was no preference in their subcellular localization when examined with myelin, synaptosomal supernatant and total homogenate fractions from the rat cerebrum and cerebellum.

Key words: Nucleoside diphosphate kinase; Isoform; Expression vector; Enzyme localization; (Rat brain)

1. Introduction

Nucleoside diphosphate (NDP[1]) kinase (EC 2.7.4.6) catalyzes transphosphorylation of a terminal phosphate group from a nucleoside triphosphate to a nucleoside diphosphate through forming a high energy phosphorylated enzyme intermediate. It has long been believed that NDP kinase plays a 'housekeeping' role as a major component of the enzymic pathway for the syntheses of triphosphate nucleotides except ATP [1]. Extreme structural conservation of the protein primary structures from higher to lower organisms [2–7] supports its fundamental role in the cell. However, some of the recent studies suggest multi-functional and regulatory roles of this protein; for example, it plays as a possible tumor metastasis suppressor factor (nm23) [2,8–14], is essential for normal morphological development in *Drosophila melanogaster* (Awd) [14,15], functions as a transcription factor of c-*myc* gene [16] and a differenti-

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Abbreviations: NDP, nucleoside diphosphate; IEF, isoelectric focusing; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; bp, base pair(s).

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ation inhibitor factor of mouse myeloid leukemia cells (M1) [17]. In the latter case, it acts outside the cell and the enzymic activity is not required for its action, whereas in the other cases whether the enzyme activity is involved remains uncertain. On the other hand, preferential association of NDP kinase with proteins that display strict specificity for guanine nucleotides is suggested [18–26]. We propose that the enzyme may play a regulatory role as a GTP supply machinery in signal transduction pathways at the steps of heterotrimeric GTP binding (G) proteins [18,24,26].

NDP kinase consists of six subunits and displays multiple electrophoretic properties [1,27]. The finding that at least two isoforms of NDP kinase are present in higher eukaryotes including rat [28], human [29] and mouse [30] has led to the realization that NDP kinase may exist as a heterooligomeric as well as homooligomeric complex that may account for multifunctional property of the enzyme. Therefore, to understand the true role of NDP kinase in the cell it seems prerequisite to know functional as well as structural properties of each isoform. Human two isoforms are highly homologous (89% identical in amino-acid sequence) but with different electric property; NDP kinase A (nm23-H1) is more acidic than NDP kinase B (nm23-H2) [31]. Interestingly, one (nm23-H1) of the two isoforms was more drastically decreased in highly metastatic human breast cancer cells compared with the other one (nm23-H2) [29]. Antimetastatic action of the mouse homolog of nm23-H1 (nm23-1) was established in a transfection experiment [32], although whether nm23-H2 or its homolog of other species possess a similar property as well is unknown. On the other hand, our previous studies [2,28,33] demonstrated that expression of the α- and β-isoforms of rat NDP kinase as shown by their transcription products is regulated independently; the α-isoform (nm23-H2 homolog), though its expression varied from one tissue to another, was the major form in most tissues except cerebrum and testis in which expression of the β-isoform (nm23-H1 homolog) was equivalent to that of the α-isoform. These observations suggest that, although the two isoforms of NDP kinase structurally resemble each other, they may possess independent role in the cell.

In the present study, in order to understand the biochemical properties of NDP kinase isoforms, rat NDP kinase α- and β-isoforms cDNAs were inserted in an expression vector pET3b and the recombinant proteins overproduced in bacteria were purified and characterized. They showed similar but not identical enzymic and physicochemical properties. Also, generation of monoclonal antibodies and their application to analyses of tissue distribution of the native enzymes revealed that the two isoform proteins are expressed in a tissue specific manner.

2. Experimental procedures

Materials. Unless otherwise mentioned, all of the reagents, enzymes, vectors, and radioisotopes used were as described previously [2,28,33].

Construction of plasmids for overproduction of NDP kinase-α and -β. A plasmid for NDP kinase α was constructed as follows. The pBluescript KS$^+$ plasmid harbouring 622 bp NDP kinase α cDNA was cleaved at an *Acc*I site in the multicloning site. The ends were converted to blunt ends by the treatment with Klenow fragment. After attachment of synthetic *Bam*HI linkers, the plasmid was digested with *Bam*HI to liberate the cDNA insert. The insert was ligated to the *Bam*HI site of pET3b expression vector [34]. The constructs were used to transform XL1-blue. A plasmid DNA with the insert in the correct orientation to T7 promoter was selected. It was digested with *Nco*I and *Nde*I after methylation with methylase *Hae*III to protect an internal *Nco*I site of the cDNA. The two sites were ligated with blunt-end ligation to yield overexpression plasmid of NDP kinase α cDNA.

In order to construct an overexpression plasmid for NDP kinase β, the pBluescript KS$^+$ plasmid harbouring 746 bp NDP kinase β cDNA was cleaved at a *Nco*I site corresponding to the translation initiation site, converted to blunt ends and then cleaved at a *Bgl*II site in the 3′-flanking region to isolate the cDNA insert. The insert was ligated to pET3b vector which had been digested with *Nde*I, converted to blunt ends and cleaved with *Bam*HI. The plasmids were then used to transform BL21(DE3)pLysS (34) for overexpression of NDP kinases.

Preparation of the cell extracts. The *E. coli* strain BL21(DE3)pLysS harboring pET3b-NDPK α or β cDNA was precultured in M9-ZB medium at 37°C overnight. Next day, the culture was diluted to A_{600} = 0.1 with the medium. When the value of A_{600} became between 0.5 and 0.6, production of NDP kinases was induced by adding 1 M IPTG (0.5 ml/500 ml culture medium) to the culture medium. After 5 h, the bacteria were harvested by centrifugation at $1400 \times g$ for 15 min at 4°C. All the following procedures to obtain the crude enzyme were performed on ice unless otherwise described. The bacteria were suspended in a lysis buffer (3 ml/g cells) containing 1 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, supplemented with phenylmethylsulfonyl fluoride (PMSF) (at final concentration of 0.1 mM) and lysozyme (800 μg/g cells) and then incubated for 20 min with occasional stirring. Thereafter deoxycholic acid (4 mg/g cells) was added while stirring continuously. The solution was placed at 37°C until the lysate became viscous. Subsequently, 1 mg/ml DNAaseI (20 ml/g cells) was added and placed at room temperature until it is no longer viscous (about 30 min). The cell lysate was centrifuged at $16000 \times g$

for 15 min at 4°C and the enzyme activity was recovered in the supernatant of the cell lysis solution. The solution was stored at −70°C until use.

Purification of recombinant NDP kinase α and β. (a) NDP kinase α: The crude enzyme prepared as described in Section 2.3 was precipitated by ammonium sulfate between 50–75% saturation. The precipitate was dissolved in buffer A (10 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.25 mM PMSF (pH 7.4)), followed by dialysis against buffer A and then applied to a DEAE-Sepharose column (5 × 14 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0–300 mM) in buffer A at a flow rate of 100 ml/h. The enzyme activity was recovered in the flow-through fractions. The fractions were pooled and applied on a Bio-Gel HTP column (5 × 14 cm) at a flow rate of 100 ml/h. The column was washed with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTT and 0.25 mM PMSF. The enzyme was eluted with a linear gradient of potassium phosphate (10–400 mM) buffer containing 1 mM DTT and 0.25 mM PMSF. The enzyme fractions were concentrated by Centriprep-10 (Amicon), and then chromatographed on a Superose 12 prepgrade column (1.6 × 50 cm, Pharmacia) equilibrated with buffer A containing 0.1 M NaCl at a flow rate of 1 ml/min. The fractions containing the enzyme were pooled, concentrated by Centriprep-10 and stored at −70°C.

(b) NDP kinase β: The crude enzyme solution prepared as described above was subjected to ammonium sulfate precipitation between 45–80% saturation. After the precipitate was dissolved in buffer A followed by dialysis against the same buffer, the enzyme solution was chromatographed successively on DEAE Sepharose (5 × 14 cm) and Bio-Gel HTP (2 × 90 cm) columns as described for NDP kinase α except that prior to chromatography on the Bio-Gel HTP column, the sample buffer was changed to 10 mM potassium phosphate, 1 mM DTT, 0.25 mM PMSF by repeated concentration and dilution. After the HTP column chromatography, the sample buffer was changed to buffer A containing 1.6 M ammonium sulfate and then chromatographed on a Phenyl Superose column (0.5 × 5 cm, Pharmacia) at a flow rate of 0.4 ml/min with a linear decreasing gradient of ammonium sulfate (1.6–0 M) in buffer A. The fractions containing the enzyme activities were pooled, the dissolving buffer was changed to 0.1 M NaCl in buffer A, and then the purified enzyme was stored at −70°C.

Enzyme assay. The enzyme assay was carried out using either the pyruvate kinase-lactate dehydrogenase coupled enzyme reaction or radioisotopic method as previously described (35).

Gel electrophoresis. For SDS-PAGE, samples were dissolved in Laemmli's sample buffer [36], boiled and applied to a gel of 14% acrylamide. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (0.25% Coomassie brilliant blue R-250, 50% trichloroacetic acid). For isoelectric focusing gel electrophoresis, final concentration of urea in the sample solution was adjusted to 7 M. After the solution was mixed with 60% glycerol-4% ampholine (pH 3.57–10) solution (1 : 1), it was applied onto a separation gel consisting of 5% acrylamide, 0.25% *N,N'*-methylene-bis acrylamide, 0.5% ribofravin and 7 M urea in the presence of either 2% ampholine (pH 3.5–10) (for staining) or 1% ampholine (pH 3.5–10) plus 1% ampholine (pH 5–8) (for immunoblotting). Isoelectric focusing was performed at 200 V for 6.5 h. The gel was stained with Coomassie brilliant blue G-250 (0.25% Coomassie brilliant blue G-250, 12.5% trichloroacetic acid).

Development of monoclonal antibodies. Balb/c mice were immunized by intraperitoneal injection of 30 mg purified protein mixed with aluminum gel and *Bordetella pertussis*, and were given three booster injections of the antigen at intervals of one week. The mice which showed a high serum reactivity to the antigen were sacrificed 4 days after the last booster injection. The spleen cells were fused with a murine myeloma cell line P3-X63.Ag-U1 using polyethylene glycol-1000. The fused cells were suspended in hypoxanthine/aminopterine/thymidine medium and distributed into 96-well culture plates. The cells were cultured for 10–12 days and their supernatants were analyzed for antibody production by ELISA and subsequently cloned twice by limiting dilution. The antibodies were purified either by gel filtration column chromatography or protein A-conjugated affinity chromatography.

Immunoblotting. After isoelectric forcusing, gel was soaked in a transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3) for 10 min to remove ampholine, and then proteins were transferred electrophoretically (at 0.1 A for 120 min at 0°C) to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 0.2 mm). Immunodetection was performed essentially as described previously [35] using either monoclonal or polyclonal anti-NDP kinase antibodies as the first antibody. Detection was done with an ECL Western blotting detection kit (Amersham) by exposing the membrane to an X-ray film (XAR, Kodak).

Miscellaneous procedures. Protein concentration was determined using a BCA Protein Assay Reagent (Pierce). The tissue extracts of rat brain, liver, heart and testis were prepared by homogenization followed by centrifugation at 105 000 × *g* for 1 h. Homogenization and preparation of myelin and synaptosomal supernatant fractions from rat cerebrum and cerebellum were carried out as described previously [37,38]. Peptides were synthesized by the solid-phase synthesis method originally described by Merrifield [39] using a peptide synthesizer (430A, Applied Biosystem, USA).

Table 1
Purification of recombinant rat NDP kinase α and β isoforms

Purification step	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1})	Purification factor	Total activity [a] (mmol min^{-1})	Recovery (%)
α-isoform					
Cell lysate supernant	826	300	1.0	250	100
$(NH_4)_2SO_4$ (50–75%)	239	690	2.3	170	68
DEAE-Sepharose	99.1	1600	5.2	160	64
Bio-Gel HTP	95.5	1100	3.7	110	44
Superose-12	47.3	1800	5.9	85	35
β-isoform					
Cell lysate supernatant	339	680	1.0	230	100
$(NH_4)_2SO_4$ (45–80%)	157	920	1.4	140	60
DEAE-Sepharose	65.5	1200	1.8	79	34
Bio-Gel HTP	52.2	880	1.3	46	19
Phenyl-Superose	12.6	1300	1.9	16	6

[a] The enzyme activity was determined by pyruvate kinase-lactate dehydrogenase coupled method.

General methods for gene technology, unless otherwise specified, were used according to the description elsewhere [40].

3. Results

Expression and purification of recombinant NDP kinase α and β

Expression of the NDP kinase α- and β-isoforms in *E. coli* induced by the addition of IPTG was evident within 2 h incubation and reached maximum after 5 h (data not shown). These enzymes produced in bacteria constituted approx. 20–50% of the total protein present in cell extracts (see Table 1). The recombinant enzymes were purified by three successive column chromatographies after ammonium sulfate precipitation as described in Section 2. In the step of DEAE-Sepharose column chromatography, the α-isoform was eluted in unretained fractions, whereas the β-isoform was retained under the same condition (pH 7.4) (data not shown), suggesting their difference in isoelectric point. The α-isoform was purified to apparent homogeneity after the third column chromatography on a Superose 12 prepgrade, whereas the β-isoform required Phenyl Superose column chromatography to be purified. Minor component(s) having molecular size of ≈ 10 kDa copurified with the β-isoform throughout was removed by the final purification step, suggesting that the minor contaminant could be derived from

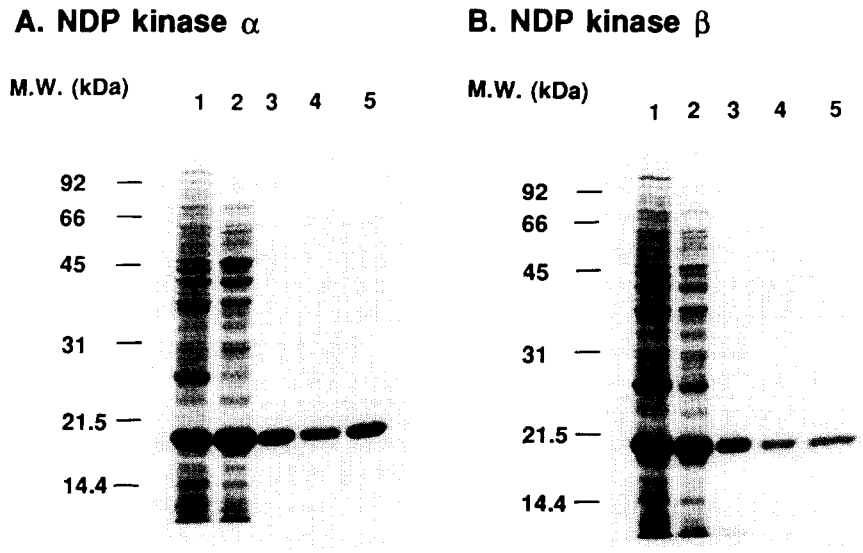

Fig. 1. SDS-PAGE of samples from each purification step of recombinant NDP kinase α (A) and β (B) isoforms. (A) Lane 1, cell lysate supernatant (76 μg); lane 2, ammonium sulfate fraction (48 μg); lane 3, DEAE Sepharose fraction (8 μg); lane 4, Bio-Gel HTP fraction (5 μg); and lane 5, Superose 12 fraction (6 μg). (B) Lane 1, cell lysate supernatant (68 μg); lane 2, ammonium sulfate fraction (37 μg); lane 3, DEAE Sepharose fraction (6 μg); lane 4, Bio-Gel HTP fraction (3.5 μg); and lane 5, Phenyl Superose fraction (4 μg).

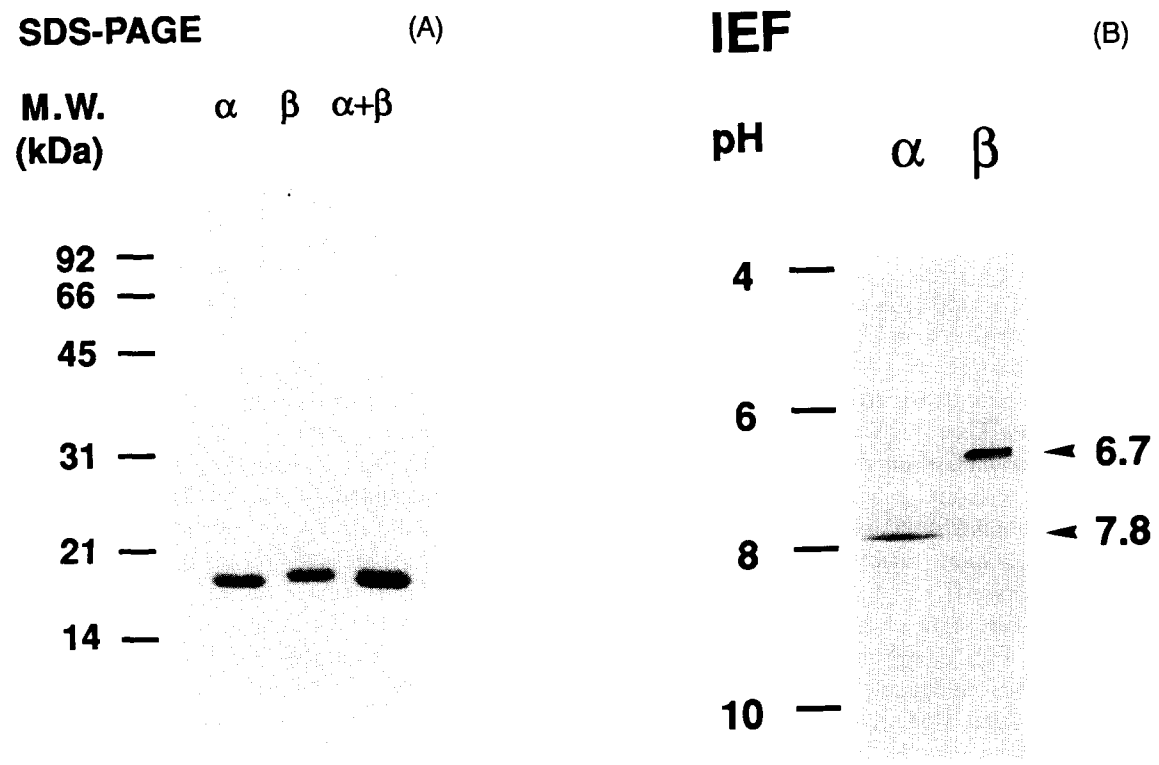

Fig. 2. Electrophoretic pattern of recombinant NDP kinase α and β-isoforms on SDS-polyacrylamide (A) and isoelectric focusing (B) gels. (A) Lane 1, recombinant NDP kinase α (1 μg); lane 2, recombinant NDP kinase β (1 μg); lane 3, mixture of recombinant NDP kinase α (1 μg) and β (1 μg). (B) Isoelectric focusing was performed on a 5% gel. Lane 1, recombinant NDP kinase α (8 μg); lane 2, recombinant NDP kinase β (8 μg).

either proteolytic cleavage of the enzyme or incomplete transcription of the expression construct.

The representative data of the purification and yield of the NDP kinase α and β are summarized in Table 1. Protein profiles of each purification step examined with SDS-PAGE is shown in Fig. 1. Specific activities and purification factor of both emzymes in the Bio-Gel HTP fraction gave lower values than those of the previous steps (Table 1). This was attributed to the carry-over in the NDP kinase assay of phosphate ions used as the elution buffer for Bio-Gel HTP column chromatography. Low yield of the β-isoform compared to the α isoform was caused by different retaining efficiency on a Centriprep-10 filter used for concentrating samples at each purification step.

Comparison of physical properties

The recombinant rat NDP kinase α- and β-isoforms have calculated molecular weight of 17283 and 17192, respectively. When their electrophoretic profiles were

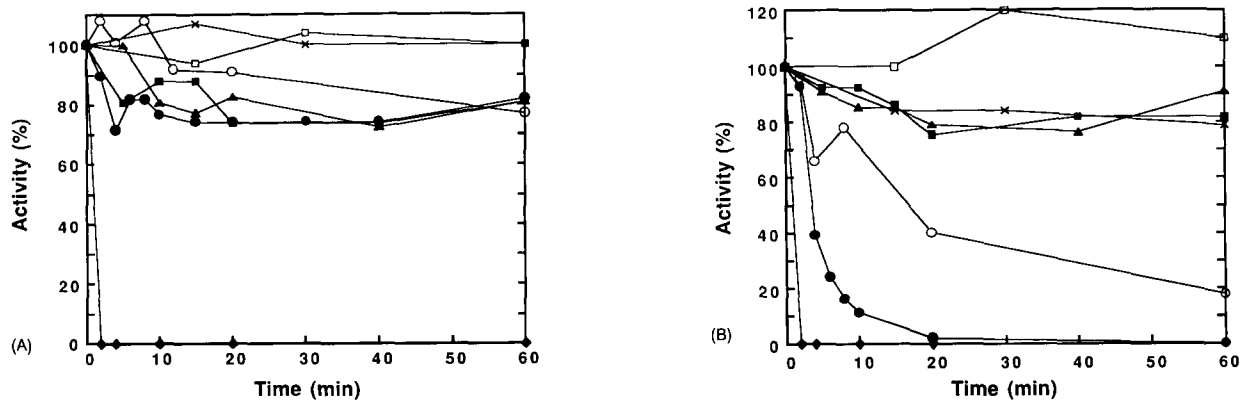

Fig. 3. Thermal stability of recombinant NDP kinase α (A) and b (B). Each isoform was incubated at 4°C (×), 20°C (▲), 37°C (■), 50°C (●) and 70°C (♦) at a protein concentration of 0.01 mg/ml or 37°C (□) and 50°C (○) at 0.1 mg/ml for indicated duration, cooled on ice and then enzyme activity was measured after appropriate dilution of each sample using the coupled enzyme method.

determined by SDS-PAGE, the α-isoform displayed somewhat faster mobility than the β-isoform (Fig. 2A). The two enzymes were satisfactorily separated by isoelectric focusing gel electrophoresis (Fig. 2B) as expected from different elution behavior on an ion-exchange column chromatography as described above. This separation was complete when isoelectric focusing gel electrophoresis was done under the denaturation condition in the presence of 7 M urea. The p*I* values of the recombinant α- and β-isoforms thus determined were 7.8 and 6.7, respectively. In the absence of urea both isoforms yielded several bands probably due to tendency to aggregate near their isoelectric points. When both isoforms were mixed and treated with 7 M urea, followed by renaturation and isoelectric focusing gel electrophoresis without urea, multiple diffuse bands instead of discrete bands appeared, suggesting heterologous hexameric complex formation as previously reported (data not shown).

These two isoforms showed different thermal stability. As shown in Fig. 3, when both isoforms were incubated at various temperatures for the indicated periods, the α-isoform was inactivated only at 70°C during the incubation time tested (Fig. 3A). But this was in good contrast with the β-isoform which was more sensitive to the temperature and inactivated gradually at 50°C within 20 min even at high protein concentration (Fig. 3B). Incubation at 37°C and lower temperatures had essentially no effect on the stability of both enzymes during the time range tested.

Comparison of enzymic properties

Substrate specificity of NDP kinase α and β for phosphate donors as well as phosphate acceptors is shown in Table 2. When phosphate acceptors were examined with 1 mM ATP, both isoforms showed lower apparent K_m and V_{max} values for GDP compared with ADP. On the other hand, when various triphosphate nucleotides were tested with 0.1 mM GDP as a phosphate acceptor, both isoforms demonstrated lower apparent K_m values for purine nucleotides than for pyrimidine nucleotides. Further, the lowest apparent K_m values were obtained with GTP for both isoforms.

While the apparent K_m values toward purine nucleoside triphosphates of the β-isoform were more than twice as much as those of the α-isoform, those toward pyrimidine nucleoside triphosphates were quite similar between them. There was no essential difference in the apparent K_m and V_{max} for diphosphate nucleotides examined.

Table 2
Apparent K_m and V_{max} for recombinant rat NDP kinase α and β with various substrates

Substrate	Rat NDPK-α		Rat NDPK-β	
	K_m (mM)	V_{max} (mmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (mmol min^{-1} mg^{-1})
ATP	1.80	4.20	4.60	3.70
GTP	0.15	0.67	0.64	0.47
UTP	27.00	9.20	16.00	2.90
CTP	10.00	1.70	12.00	0.84
GDP	0.049	1.41	0.041	1.04
ADP	0.066	2.65	0.080	2.35

[a] The enzyme activity was determined by radioisotopic method using either 0.1 mM ^{3}H-GDP with varying amount of unlabeled triphosphate nucleotides, or varying amount of ^{3}H-GDP or ^{3}H-ADP with 1 mM ATP.

Monoclonal antibodies and distribution of NDP kinase isoforms in rat tissues

In an attempt to identify each isoform in cells and tissues and speculate their roles, monoclonal antibodies that recognize each isoform were prepared using as antigens purified rat liver cytosolic enzyme, recombinant rat NDP kinase α and β, and peptides corresponding to the variable portion of the two isoforms (amino-acid sequence 37–50; see Ref. 28). Four clones (KM802, KM809, KM810, KM818) that secreted antibodies only reactive to recombinant α isoform by ELISA were isolated. The specificities of these monoclonal antibodies were confirmed by Western blot analysis as well (Fig. 4). We also obtained clones that secreted antibodies reactive to both α- and β-isoforms, but unfortunately those antibodies also reacted with some other proteins nonspecifically. Isolation of an antibody reactive to only β-isoform was unsuccessful. The peptide antigens were essentially inert for antibody production. A polyclonal antibody (NK-2) produced against the rat liver cytosolic NDP kinase [35] crossreacted with both isoforms (Fig. 4). Reactivity of the polyclonal and monoclonal antibodies were unaffected by the presence of 7 M urea.

In a previous study [28] we reported by measuring transcript levels that expression of rat NDP kinase isoforms encoded by different genes was regulated independently and that the α-isoform was the major component in most rat tissues except brain and testis. In the latter tissues the β-isoform was expressed to an extent comparable to that of the α-isoform. In order to confirm this tendency at the protein level, we examined tissue extracts by SDS-PAGE followed by Western blotting. The results demonstrated that the polyclonal (NK-2) and monoclonal (KM809) antibodies specifically reacted with 18 kDa protein(s) (Fig. 5). Isoelectric focusing gel electrophoresis revealed that the major band (p*I* 7.2) was found to be the α isoform because of its reactivity with KM809 (Fig. 6). NK-2 identified several bands other than the α isoform. Among them, the protein band in the highest position (acidic, p*I* 6.1) seen in the brain and testis but not in the liver and heart samples was assigned to the β-isoform in view of the lack of reactivity with KM809 in conjunction with

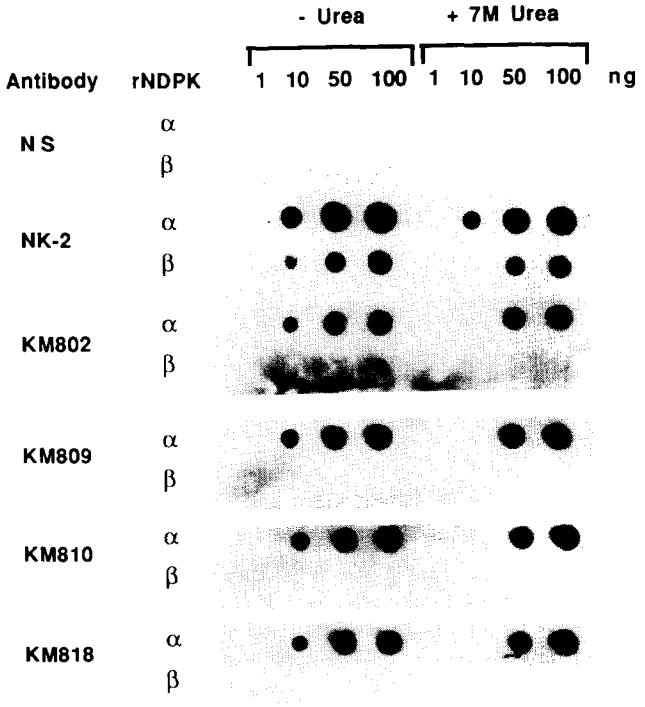

Fig. 4. Crossreactivity of polyclonal and monoclonal antibodies to recombinant NDP kinase α and β. Recombinant isoforms (rNDPK) were dissolved in the absence and presence of 7 M urea and then each sample, at indicated amounts, was applied onto PVDF membranes. The membranes were then treated with either nonimmune rabbit serum (NS), affinity purified polyclonal anti-rat NDP kinase rabbit IgG (NK-2) or one of monoclonal anti-rat NDP kinase mouse immunoglobulins (KM802, IgM subclass; KM809, KM810, KM818, IgG1 subclass).

the data of RNA expression levels of the two isoforms in a previous study [28]. Under these conditions, neither nonimmune rabbit serum (see Ref. 35) nor nonimmune mouse serum reacted with proteins from rat tissue extracts examined (data not shown). It should be noted that intensity of the bands does not necessarily parallel their protein amounts because, for example, the reactivity of NK-2 to the two isoforms differs several fold (see Fig. 4).

Since the rat brain extract contained relatively high amount of the β-isoform, we further examined subcellular localization of the two isoforms (Fig. 7). The relative amount of the two, however, was essentially the same in the myelin and synaptosomal supernatant fractions as in the whole homogenates from the rat cerebrum and cerebellum.

4. Discussion

There has been an argument on the existence of NDP kinase isoforms. Multiple forms in their electric properties in crude tissue extracts, as well as multi-intracellular localization postulated possible presence of isoforms [1]. But later studies [35,41] with purified preparations demonstrated data against the proposal and showed the possibility that the multiplicity could have resulted from various extent of phosphorylated intermediate formation of subunits of hexameric NDP kinase. In contrast, recent cDNA cloning studies revealed unequivocally that there are at least two isoforms in higher eukaryotes including human [29], rat [28] and mouse [30] and this conclusion was supported by a peptide analysis of human erythrocyte enzymes [31]. Although there is a striking conservation between the two isoforms in amino-acid sequence, some studies suggest their independent roles in relation to tumor metastasis [29] and tissue expression [2,28]. In the present study recombinant rat NDP kinase α- and β-isoforms showed similar but not identical substrate specificity with different thermal stability and isoelectric point. Further, tissue dependent expression of the two isoforms was demonstrated in rat at the protein level by an immunological method in combination with isoelectric focusing gel electrophoresis.

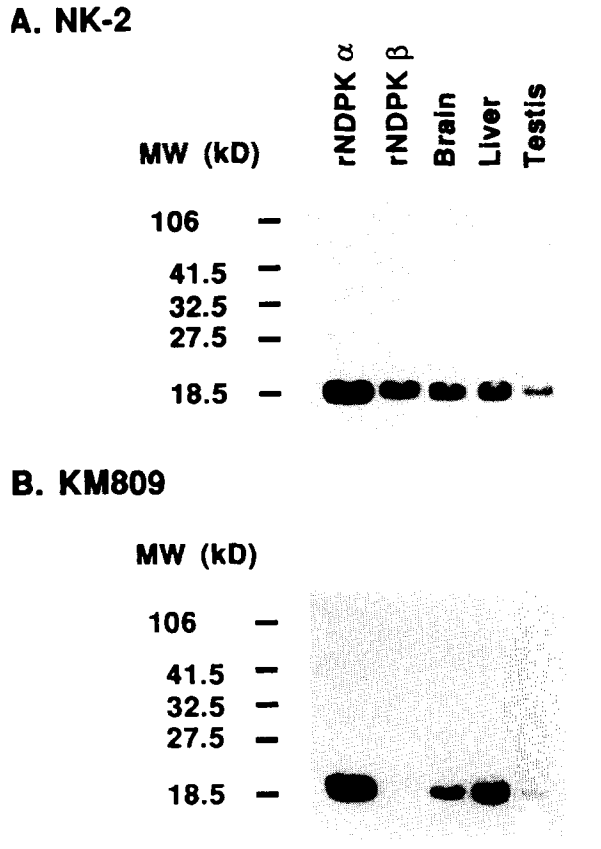

Fig. 5. Specificity of polyclonal and monoclonal antibodies for tissue-derived NDP kinases. Tissue extracts (15 μg protein each) from a rat in parallel with recombinant NDP kinase (rNDPK) α and β (0.36 μg each) were separated by SDS-PAGE, proteins separated were transferred onto PVDF membranes and then the membranes were treated with either polyclonal (NK-2) (A) or monoclonal (KM809) (B) antibodies.

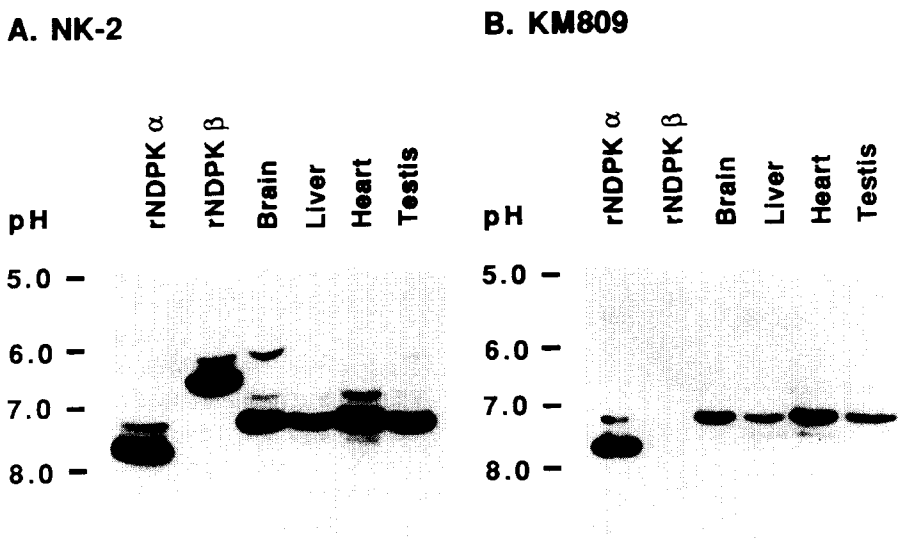

Fig. 6. Demonstration of NDP kinase isoforms in rat tissues after isoelectric focusing gel electrophoresis using polyclonal (A) and monoclonal (B) antibodies. Tissue extracts from a rat (20 μg and 30 μg protein for each lane in (A) and (B), respectively) in parallel with recombinant NDP kinase (rNDPK) α and β (0.36 μg each in (A) and 0.72 μg each in (B)) were separated by isoelectric focusing gel electrophoresis, proteins separated were electrophoretically transferred onto PVDF membranes and then the membranes were treated with either polyclonal (NK-2) (A) or monoclonal (KM809) (B) antibodies.

Amino-acid sequence of the recombinant NDP kinase α and β starts from methionine, whereas native enzymes suffer proteolytic cleavage followed by modification at the α-NH_2 group of the newly produced NH_2-terminal amino-acid residue [2,31]. The NH_2-terminal amino acid of the native enzyme from the rat liver cytosol was tentatively identified as Arg-6 by peptide analyses after acylamino-acid-releasing enzyme treatment [2]. Despite these difference in the structure of the NH_2-terminal ends between native and recombinant enzymes, hexameric structure of recombinant emzymes seemed to be well conserved judging from an

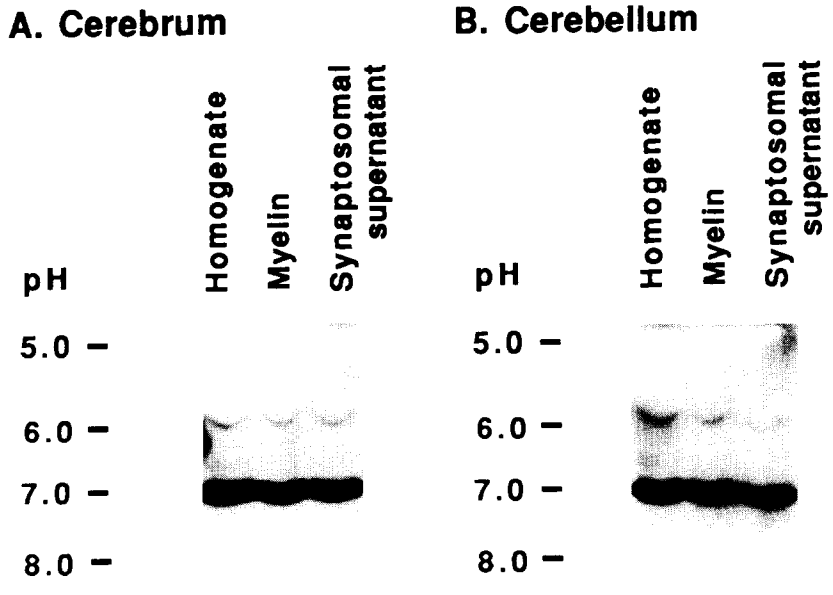

Fig. 7. Subcellular localization of NDP kinase isoforms in rat cerebrum (A) and cerebellum (B). Homogenates, myelin and synaptosomal supernatant fractions were prepared as described in the text. The fractions (15 μg each) were separated by isoelectric focusig gel electrophoresis, followed by Western blotting analysis using polyclonal antibody (NK-2).

elution pattern on a gel filtration column chromatography (data not shown). Moreover, specific activities of the purified recombinant NDP kinase α- and β-isoforms displayed similar values to those of tissue-derived purified enzymes [35]. Therefore, the recombinant enzymes seem to retain most of characteristics of the native ones although their fine differences remain to be studied.

When amino-acid sequence of the two isoforms was compared, there were two variable portions located at NH_2-terminal third and the COOH-terminus [28]. The former portion of the α isoform contains a peptide sequence (AMKXL) known as an ATP binding consensus sequence of cyclic nucleotide-dependent protein kinases [42,43]. Interestingly, as observed in the protein kinases, this sequence was accompanied in its NH_2-terminal side by a sequence like glycine rich loop known as a phosphoryl binding domain [44]. This hypothetical ATP binding sequence is broken in the β-isoform. From a recent X-ray crystallographic study of *Dictyostelium discoideum* NDP kinase [27], this peptide portion was found to be located in $\beta 2$ strand between loop 51–59 and helix $\alpha 4$, both of which form a cleft accessible from the outside as a possible binding site of nucleotides. Therefore, it was anticipated that the two isoforms may display different substrate specificity. The data in the present study demonstrated that the α-isoform showed lower apparent K_m values for ATP and GTP than did the β-isoform with no essential difference for other nucleotides tested (Table 2), making it plausible that the peptide sequence may be responsible for substrate binding. However, since the substrate specificity seemed not so strict, the role played by this AMKXL sequence remains to be investigated further.

The role of the COOH-terminal variable portion also remains unclear. This sequence extends to the protein surface on the periphery of the hexamer [27], suggesting that this portion may determine interaction of NDP kinase with other proteins such as nucleotide-binding proteins. It, therefore, cannot be ruled out that the two isoforms may not profoundly differ in their catalytic property but in their preference in interacting with other proteins.

As observed with human NDP kinases, the two recombinant isoforms showed different isoelectric points (the α-isoform, p*I* 7.8, and the β-isoform, p*I* 6.7), both of which were somewhat higher than those of the corresponding native enzymes (7.2 versus 6.1). The p*I* difference between native and recombinant enzymes is presumably ascribed to the processing of immature forms to the mature enzymes. The p*I* (7.2) of the native α isoform determined in this study was higher than the reported value (6.0) of the rat liver cytosolic NDP kinase [35] which was found to consist of mostly the α isoform in this study. At present we have no discrete answer for the discrepancy but it might have arisen from different experimental conditions (e.g., undenatured versus denatured ones) in which p*I* was measured.

In order to speculate the role of NDP kinase isoforms, knowledge of their distribution in cells and tissues are desirable. In our previous study [28], it was demonstrated that distribution of the two isoforms mRNA was different from one tissue to another; in general, the α-isoform was the major form in rat except brain and testis. This was confirmed at the protein level in this study by performing isoelectric focusing gel electrophoresis followed by immunoblotting. Further, there existed no preferential subcellular localization of the two isoforms when examined with myelin and synaptosomal supernatant fractions. It should be recalled that a few unidentified bands other than the major α- and β-isoforms were detected by isoelectric focusing gel electrophoresis. Whether these were derived from either the α- or β-isoform through modification by such as a proteolytic cleavage, or they were the products of related but different genes remains to be determined.

What is the physiological role of each isoform? Although there are some differences in the substrate specificity between the two isoforms as described above, it seems difficult to explain 'multi-functional' properties of NDP kinase from such data. The difference in heat stability may be another clue to speculate their roles because the stability depends on protein folding in monomer and/or subunit interaction. A recent paper [45] reported that a single amino-acid substitution (proline to serine) of *Drosophila* NDP kinase in Kpn loop which was implicated in trimer contacts in a hexameric structure resulted in reduced stability to denaturation by heat and urea with no appreciable change in catalytic function. This mutation alone is nonlethal, but it provides a lethal effect when accompanied by a second mutation in the *prune* locus, the gene which is assumed to code for a GTPase activating protein (GAP) homolog [25]. In the present case, however, there was no difference in this region between the α- and β-isoforms. Further studies are required to identify the specific physiological function of each NDP kinase isoform in the cell.

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