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Membrane-associated Nucleoside Diphosphate Kinase from Rat Liver

PURIFICATION, CHARACTERIZATION, AND COMPARISON WITH CYTOSOLIC ENZYME*

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Narimichi Kimura and Nobuko Shimada

From the Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, 35-2, Sakaecho, Itabashiku, Tokyo-173, Japan

Previous studies from this laboratory have proposed that membrane-associated nucleoside diphosphate kinase (m-NDP kinase) may play a role in regulation of adenylate cyclase by channeling GTP, an essential cofactor of adenylate cyclase regulation, into GTP-binding protein (G_s) in a hormone-dependent manner. To understand the true role of m-NDP kinase, in the present study, the m-NDP kinase was solubilized and purified to apparent homogeneity from rat liver purified plasma membranes and characterized in comparison with the cytosolic enzyme purified from the same tissue (s-NDP kinase). Some physical properties determined were: molecular weight (monomer), 18,300; sedimentation coefficient ($s_{20,w}$), 6.2 S; isoelectric point (pI), 6.0. These values and kinetic parameters of the m-NDP kinase were almost identical to those of the s-NDP kinase whose characteristics were more extensively studied. A peptide mapping study of the ^{125}I-labeled m- and s-NDP kinases gave essentially identical patterns. Polyclonal antibodies against the s-NDP kinase, which also cross-reacted with the m-NDP kinase, were prepared. Immunoblotting studies with the affinity-purified antibodies revealed that the monomer molecular weight of the purified m- and s-NDP kinases was identical to the values of unpurified enzymes present in membranes and crude extract. These results demonstrate that the purified m-NDP kinase underwent no remarkable modification during solubilization and purification, and that the m- and s-NDP kinases are quite similar in protein structure, if at all different. The physiological relevance of the m-NDP kinase in relation to the adenylate cyclase system is discussed.

Nucleosidediphosphate kinase (NDP kinase, EC 2.7.4.6.)[1] catalyzes a transfer of the terminal phosphate group of 5′-triphosphate nucleotides to 5′-diphosphate nucleotides by the following mechanism involving a high energy phosphorylated enzyme intermediate.

$$N_1TP + E = E\text{-}P + N_1DP \quad (1)$$

$$N_2DP + E\text{-}P = E + N_2TP \quad (2)$$

N_1 and N_2 can be any ribo- or deoxyribonucleosides with purine or pyrimidine base structures.

The enzyme, first reported in 1953 (Krebs and Hems, 1953; Berg and Joklik, 1953), has been purified from various sources to apparent homogeneity, and its structural and physicochemical properties have been elucidated (Pedersen, 1968; Colomb *et al.*, 1969; Colomb *et al.*, 1972; Palmieri *et al.*, 1973; Robinson *et al.*, 1981; Nickerson and Wells, 1984). It is generally accepted that NDP kinase is a major component of the enzymatic pathway for the synthesis of nucleoside triphosphates other than ATP. However, there has been uncertainty on the regulatory role of the enzyme since the activity of NDP kinases in many tissues is relatively high and the enzymes display low specificity for nucleotide substrates (Parks and Agarwal, 1973).

Hormone-sensitive adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1.) is a transmembrane-signaling machinery which has been a target of extensive studies. Most of the adenylate cyclase systems from various sources consist of at least three components, hormone receptor, guanine nucleotide-binding proteins (G_s, G_i), and catalyst (Gilman, 1984). GTP, a cytosolic cofactor, is absolutely essential for hormone-dependent regulation of the enzyme (Kimura and Nagata, 1977; Rodbell *et al.*, 1971). In these enzyme systems, we have recently demonstrated that membrane-associated (m-) NDP kinase, in addition to the three known components, may play a role in regulation of adenylate cyclase by channeling GTP, a reaction product of the m-NDP kinase, into the G_s in a hormone-dependent manner (Kimura and Nagata, 1979; Kimura and Shimada, 1983; Kimura and Johnson, 1983; Kimura and Shimada, 1985; Kimura and Shimada, 1986). The following observations support this view. 1) In stimulatory regulation of adenylate cyclase systems by glucagon as well as other hormones, it has been observed that GDP is as effective as GTP in supporting hormonal activation of the enzyme under the assay condition devoid of an ATP regenerating system (Rodbell *et al.*, 1971; Harwood *et al.*, 1973; Kimura and Nagata, 1977; Iyenger *et al.*, 1980; Totsuka *et al.*, 1982; Kimura *et al.*, 1985). 2) This activation by hormone with added GDP was found to occur due to a small amount of GTP formed by the action of m-NDP kinase (Kimura and Nagata, 1979; Kimura and Shimada, 1983; Murphy and Stansfield, 1983; Kimura et al., 1985), and GDP itself was shown to be a strong competitive inhibitor for GTP (Kimura and Shimada, 1983). 3) In the case of adenylate cyclase activation

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[1] The abbreviations used are: NDP kinase, nucleosidediphosphate kinase; m- and s-NDP kinases, membrane-associated and cytosolic NDP kinases, respectively; G_s and G_i, the stimulatory and inhibitory GTP-binding proteins of adenylate cyclase system, respectively; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; SV 40, simian virus 40; NRK, normal rat kidney; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

TABLE 1

Purification of m-NDP kinase from rat liver purified plasma membranes

Purification step	Total protein	Specific activity	Purification	Total activity	Recovery
	mg	*μmol/min/mg*	*-fold*	*μmol/min*	*%*
CHAPS extract	320	0.21	1.0	65.6	100
Blue Sepharose	19.2	2.08	9.9	39.9	60.8
DEAE-Sepharose	2.84	8.78	41.8	24.9	38.0
Ultrogel AcA 34				16.8	25.6
Bio-Gel HTP				3.87	5.9
Ultrogel AcA 34	0.001[a]	2800[a]	13000[a]	2.72	4.1

[a] These values were calculated using the protein amount estimated from a silver-stained band after SDS-polyacrylamide gel electrophoresis.

by cholera toxin, it catalyzes ADP-ribosylation of the G_s by participation with ADP-ribosylation factor, another membrane-associated GTP-binding protein (Kahn and Gilman, 1984). In addition to serving as a cofactor in the ADP-ribosylation reaction, GTP is required to stabilize the high activity state of the ADP-ribosylated adenylate cyclase (Shimada and Kimura, 1983). However, neither of these two GTP-dependent processes occurred with added GDP under the condition where GTP production from the added GDP proceeded equally (Kimura and Shimada, 1985). These observations at least demonstrate that adenylate cyclase activity can be regulated by microenvironmental GTP surrounding the G_s and raise the possibility that the m-NDP kinase could be a factor to affect this GTP pool, although the physiological significance and the underlying mechanism of this phenomenon remain to be determined.

To understand the true role of m-NDP kinase in hormone-sensitive adenylate cyclase, we undertook, in the present study, purification and characterization of the m-NDP kinase present in rat liver plasma membranes and compared it with the cytosolic enzyme (s-NDP kinase) from the same tissue.

MATERIALS AND METHODS[2]

RESULTS

Purification of m-NDP Kinase—NDP kinase is an enzyme which can be retained by an anionic dye, blue Sepharose affinity column in the presence of nonionic detergents, although the majority of proteins in nonionic detergent extract is not retained (Robinson *et al.*, 1980). In the present study, most of the m-NDP kinase activity extracted with 10 mM CHAPS from plasma membranes was retained by the blue Sepharose column. It was of interest, however, that an elution buffer containing a high concentration of salts (0.5 M NaCl and 1 M Tris/HCl) along with 2 mM ATP (Fig. 1*A*) was required to detach the m-NDP kinase effectively from the column compared with the elution of the s-NDP kinase (0.3 M NaCl) (Fig. 1*B*). For example, 0.8 M NaCl along with 5 mM ATP in TMEDP, 1 mM CHAPS was insufficient for complete elution of the m-NDP kinase. Linear salt gradient elution of m- and s-NDP kinases of the blue Sepharose step from DEAE-Sepharose column resolved these enzyme activities at least into four peaks in both cases, the concentration of NaCl corresponding to each peak being 0, 15, 40, and 60 mM, in either case (not shown). However, both enzymes obtained from the ion exchange column by stepwise elution with the buffers containing 0.1 M NaCl displayed a single isoelectric point after purification to apparent homogeneity as described below. Values of a typical purification of the m-NDP kinase are given in Table 1.

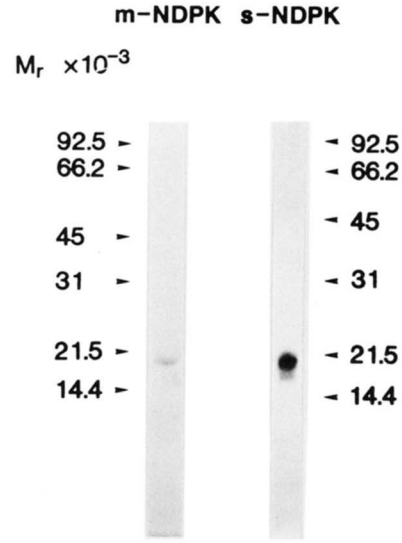

FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified m- and s-NDP kinases.** Electrophoresis was done on a 14% gel and silver-stained as described under "Materials and Methods" with 35 ng of m-NDP kinase and 320 ng of s-NDP kinase. The protein standards (Bio-Rad) used to determine molecular weight were: phosphorylase *b*, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400. *NDPK*, nucleosidediphosphate kinase.

Physical Properties of m-NDP Kinase: Comparison with Those of s-NDP Kinase—The purified m-NDP kinase showed a single band with an apparent molecular weight of 18,300 on SDS-PAGE (Fig. 2), the size being identical to that of the major band of the purified s-NDP kinase which frequently displayed a doublet of protein bands. The sedimentation coefficient of the m-NDP kinase obtained by centrifugation through 5–20% sucrose gradients in H_2O and D_2O in the presence of 0.1% Lubrol PX ($\bar{v}$ = 0.958 (Steele *et al.*, 1978)) was similar (6.2 S in H_2O and 6.3 S in D_2O), indicating that no significant binding of the detergent occurred to the m-NDP kinase, and that the partial specific volume of the m-NDP kinase is similar to that of globular proteins used as standards (~0.74). In separate experiments, the sedimentation coefficient of the s-NDP kinase was estimated to be 6.2 S. Isoelectric point of the purified m- and s-NDP kinases was determined to be 6.0 (Fig. 4). The observation of an ATP-induced shift in the isoelectric point (from 6.0 to 5.4) is probably due to the fact that these enzymes, in the presence of ATP, converted to the phosphorylated intermediate form by receiving a terminal phosphate of ATP (Walinder *et al.*, 1969; Nickerson and Wells, 1984). Table 2 shows these phys-

[2] Portions of this paper (including "Materials and Methods," Figs. 1, 3, and 4, and Tables 3 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE 2

Physical properties of purified m- and s-NDP kinases

	m-NDP kinase	s-NDP kinase
Sedimentation coefficient, $s_{20,w}$ (10^{-13} s)	6.2[a]	6.2
Stokes radius, a (nm)		3.7
Partial specific volume, $\bar{v}$ (cm^3/g)	0.74[b]	0.740[c]
Molecular weight, M_r (oligomeric form)		99,700[d]
Molecular weight, M_r (monomeric form)	18,300	18,300
Frictional ratio, f/f_0		1.11[e]
Isoelectric point, pI	6.0	6.0

[a] Since the migration of the m-NDP kinase relative to the calibrating standards was unchanged in H_2O and D_2O, the $s_{20,w}$ value determined in H_2O was chosen.

[b] The value was estimated to be identical to that of standards (~0.74) from sucrose density gradient centrifugation experiments.

[c] The value was calculated from the amino acid composition.

[d] The value was calculated from the equation

$$M_r = \frac{6\pi N\ \eta_{20,w}\ a\ s_{20,w}}{1 - \bar{v}\ \rho_{20,w}}$$

where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20 °C, $\rho_{20,w}$ is the density of water at 20 °C, and $s_{20,w}$, a, and $\bar{v}$ are the values reported above.

[e] The frictional ratio was calculated assuming a hydration of 0.2 g/g of protein, as follows:

$$f/f_0 = a\left[\frac{4\pi N}{3\ M_r(\bar{v} + (0.2/\rho_{20,w}))}\right]^{1/3}.$$

ical properties of both enzymes as well as some additional data on the s-NDP kinase.

Comparison of the Proteolytic Fragments of m- and s-NDP Kinases—The structural similarity of NDP kinase in polypeptide chain length and amino acid composition from lower to higher organisms has been described (Robinson *et al.*, 1981). Actually, the monomer molecular size (Fig. 2) and amino acid composition (Table 3) of the s-NDP kinase obtained in this study were quite similar to those from other sources (Palmieri *et al.*, 1973; Robinson *et al.*, 1981; Koyama *et al.*, 1984; Ohtsuki *et al.*, 1984). In the light of these observations, the similarity between m- and s-NDP kinases may not be surprising. However, since immunoblotting studies demonstrated that there were differences in molecular size and cross-reactivity of the enzymes from different species (rat liver cytosol *versus* bovine liver cytosol, bakers' yeast, and *Escherichia coli*) (not shown), there could be a microheterogeneity between m- and s-NDP kinases. This possibility was tested using ^{125}I-labeled m- and s-NDP kinases by subjecting them to limited proteolysis, followed by SDS-PAGE (Cleveland *et al.*, 1977). As shown in Fig. 5, V8 protease and α-chymotrypsin treatments gave three to five discrete bands depending on the enzyme concentration used, whereas subtilisin BPN′ and papain treatments resulted in extensive cleavage with small peptides being produced. Under these conditions, there was no significant difference between m- and s-NDP kinases, suggesting that the two enzymes are quite similar, if at all different, in terms of amino acid sequence, although conclusion on whether these two are identical should be retained until elucidation of their actual primary structures.

Catalytic Properties of m- and s-NDP Kinases—Some of the kinetic parameters of m- and s-NDP kinase determined with isotopic method are given in Table 4. Among the diphosphate nucleotides tested, an apparent K_m for GDP was the lowest. The V_{max} values for m- and s-NDP kinases determined with 0.1 mM GDP as an acceptor and ATP as a varying

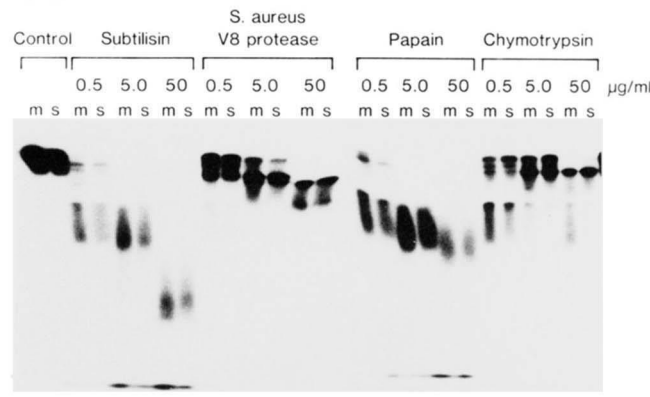

FIG. 5. **Limited proteolysis of m- and s-NDP kinases by various proteases.** ^{125}I-Radiolabeled m- and s-NDP kinases in 0.125 M Tris/HCl, pH 6.8, containing 0.1% SDS and 2 mM EDTA (in 10 μl) were mixed with 10 μl of buffer alone (0.1 M Tris/HCl) or each enzyme solution of subtilisin BPN′, *Staphylococcus aureus* V8 protease, papain, or α-chymotrypsin in the same buffer to give final concentrations as shown in the figure. Incubation was carried out for 30 min at 37 °C and stopped by 5 μl of 5 × SDS buffer (Laemmli, 1970). The samples were then subjected to SDS-PAGE with 20% gels.

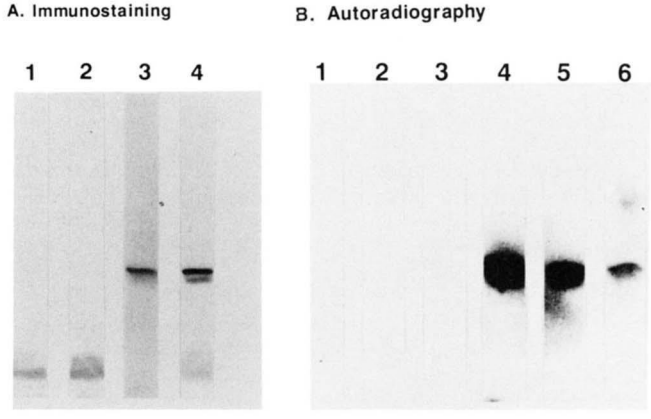

FIG. 6. **Immunoelectrophoretic blotting of the purified m- and s-NDP kinases, rat liver crude extract, and rat liver purified plasma membranes with antiserum or affinity-purified antibodies against s-NDP kinase.** *A*, purified s-NDP kinase (200 ng) (*lanes 1* and *3*) and crude extract (100 μg) (*lanes 2* and *4*) from rat liver were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The blotted proteins were reacted with nonimmune serum (*lanes 1* and *2*), antiserum (*lane 3*), or affinity-purified antibodies (*lane 4*) for 1 h at room temperature and then immunostained. *B*, purified s-NDP kinase (100 ng) (*lanes 1* and *4*), purified m-NDP kinase (70 ng) (*lanes 2* and *5*), and rat liver purified plasma membranes (180 μg) (*lanes 3* and *6*) were electrophoresed on 14% gels and transferred onto nitrocellulose membranes. The blotted proteins were reacted with nonimmune serum (*lanes 1–3*) or affinity-purified antibodies (*lanes 4–6*), followed by a second incubation with ^{125}I-Protein A. Details are given under "Materials and Methods."

phosphate donor were 0.88 and 0.86 mmol/min/mg, respectively. Diphosphate nucleotides, at high concentrations, were all inhibitory for both enzymes, probably due to an abortive complex formation with the enzymes as suggested previously (Parks and Agarwal, 1973). The apparent K_a values of the m- and s-NDP kinases for $MgCl_2$ with GDP and ATP as substrates were estimated to be 0.29 and 0.41 mM, respectively. There were essentially no differences between the two enzymes in pH optima (6.5–9.5) and dependency on various divalent cations.

Immunological Studies—Specific antibodies against the m-NDP kinase are desirable tools for elucidation of the physiological significance of this enzyme. However, they have been

unavailable since the amount of m-NDP kinase we obtained was very small. Therefore, taking advantage of the structural similarity between m- and sNDP kinases, we prepared antisera against the s-NDP kinase, which cross-reacted with the m-NDP kinase as well. From the study of enzyme-linked immunosorbent assay analysis, half-maximal titers of the antisera against the purified s-NDP kinase were about 1/2000. Nonimmune rabbit serum did not cross-react with the enzyme. Mono-specific interaction of the affinity-purified antibodies with the s-NDP kinase was demonstrated by immunoelectrophoretic blotting (Fig. 6*A*, *lanes 3* and *4*). The monospecific antibodies also cross-reacted with the purified m-NDP kinase, as well as the unpurified enzyme present in rat liver plasma membranes (Fig. 6*B*, *lanes 5* and *6*). The observation that molecular weights of the purified and unpurified m-NDP kinases were identical suggests that the m-NDP kinase, which we purified in the present study, underwent no remarkable modification during solubilization and purification steps, providing an additional support for the notion that m- and s-NDP kinases are quite similar, if not identical.

DISCUSSION

It has been accepted that NDP kinase is distributed mainly in the cytosolic fraction of cells with a small amount in other subcellular fractions such as mitochondria and nuclei (Parks and Agarwal, 1973). Although papers describing purification of the NDP kinase from particulate fractions have appeared (Robinson *et al.*, 1981; Lam and Packham, 1986), the starting materials they used were rich in mitochondria, and the physiological relevance of the enzyme remains unclear in these studies. By contrast, we have found the NDP kinase activity in purified plasma membranes intimately related to the glucagon, β-adrenergic agonist, and prostaglandin E_1-dependent regulation of adenylate cyclases from rat livers (Kimura and Nagata, 1979; Kimura and Shimada, 1983; Kimura and Shimada, 1985; Kimura and Shimada, 1986), SV 40-transformed cultured NRK cells (Kimura and Johnson, 1983), and human platelets (Kimura *et al.*, 1985), respectively. In the present study, we have purified the m-NDP kinase from rat liver purified plasma membranes to apparent homogeneity and compared it with the s-NDP kinase purified from the liver cytosol.

The purified m-NDP kinase displayed striking similarities to the cytosolic s-NDP kinase in physical, chemical, kinetical, and immunological properties with the exception that a buffer with higher ionic strength was required to elute the former enzyme from blue Sepharose column. Membrane phospholipids extracted by CHAPS along with the crude m-NDP kinase may be responsible for this difference, because the blue Sepharose step immediately followed the CHAPS extraction. Based on these data, it is assumed that the m-NDP kinase, as well as s-NDP kinase, is a pentamer or a hexamer. The purified enzymes and those in crude preparations often displayed a doublet of protein bands. However, whether these enzymes consist of homologous or heterologous subunits remained to be elucidated in future studies.

There is a possibility that m-NDP kinase could be one of the isozymes of s-NDP kinase since several papers have described electrophoretic heterogeneity of the NDP kinase from various sources (Parks and Agarwal, 1973). On the contrary, recent studies with purified enzyme preparations rather indicate monoisozymic form (Robinson *et al.*, 1981; Islam and Burns, 1984; Nickerson and Wells, 1984). In the present study, the m-NDP kinase, as well as the s-NDP kinase, displayed multiple forms on DEAE-Sepharose column chromatography by a linear salt gradient elution, whereas the major part of the two enzymes from the final purification steps showed a single isoelectric point (6.0), raising the possibility that the multiple forms of either enzyme have derived from a single form (Nickerson and Wells, 1984). It should be recalled that multiple forms of NDP kinase were reported, in most cases, for crude extracts or preparations of the early stage of enzyme purification (Parks and Agarwal, 1973; Nakamura and Sugino, 1966; Glaze and Wadkins, 1967; Edlund *et al.*, 1969; Cheng *et al.*, 1971; Cheng *et al.*, 1973; Huitorel *et al.*, 1984), whereas the monoisozymic form has been reported for the purified enzymes (Robinson *et al.*, 1981; Islam and Burns, 1984; Nickerson and Wells, 1984). Because an NDP kinase hexamer (or pentamer) could contain as many as six (or five) phosphates/one molecule and because extent of phosphorylation of the native enzyme could be affected by ATP and/or ADP levels (see Fig. 4), the occurance of multiple forms in the crude preparations is not unexpected. The present purification methods adopted the blue Sepharose step where both enzymes adsorbed to the column were eluted by buffers containing ATP, favoring formation of phosphorylated enzymes. Taking into consideration the relative instability of the phosphorylated enzyme (Koyama *et al.*, 1984), however, it can be presumed that the dephosphorylated (native) enzymes (pI 6.0) have accumulated during purification processes. Taken together, the possibility that the m-NDP kinase is one of the isozymes of s-NDP kinase seems unlikely.

The remarkable resemblance between m- and s-NDP kinases leads us to ask whether these two enzymes are actually identical and what makes these two enzymes different in intracellular distribution. Unfortunately, we have had no definite answer for these questions. The idea that several enzymes partition between kinetically distinct soluble and membrane-bound forms (ambiquitous enzymes) has been proposed (Wilson, 1978). Also, recent studies have shown that proteins can be incorporated into membranes through covalently attached small molecules as a hydrophobic anchor (Wold, 1986; Low *et al.*, 1986). These possibilities remain to be tested for the present case in future studies.

Despite the similarities between m- and s-NDP kinases, there have been observations which demonstrate a unique role of the m-NDP kinase in hormone-sensitive adenylate cyclase systems in membranes, although the molecular mechanism by which the m-NDP kinase is bound to plasma membranes remains unclear. In the SV 40-transformed NRK cells, increased m-NDP kinase activity in purified membranes, with unaltered s-NDP kinase activity in the cytosol, correlated with an enhanced guanine nucleotide-dependent adenylate cyclase activity as a possible basis for increased agonist sensitivities of the cells (Kimura and Johnson, 1983). In the glucagon-sensitive adenylate cyclase system, the membrane-associated enzyme activity allows hormone, but not cholera toxin, to activate adenylate cyclase in the presence of added GDP by channeling GTP, the reaction product of the m-NDP kinase, into the GTP-binding protein (G_s) (Kimura and Shimada, 1985). Meanwhile, the GTP produced by m-NDP kinase is not available for ADP-ribosylation factor, another membrane-associated GTP binding protein, which plays an essential role in ADP-ribosylation of the G_s by cholera toxin (Kimura and Shimada, 1986). All these phenomenological observations favor the membrane-associated form of NDP kinase intimately related to hormone-sensitive adenylate cyclase system.

Whether the m-NDP kinase has a direct interaction with any one of the components of adenylate cyclase system is a fundamental knowledge to speculate the role of the enzyme

as a GTP-channeling machinery for the adenylate cyclase system. The m-NDP kinase is present in purified rat liver plasma membranes at a concentration of approximately 1 pmol/mg of membrane protein. This value is comparable to those of high affinity glucagon receptor (1–2 pmol/mg)[3] and G_s (0.2–1 pmol/mg) (Bokoch *et al.*, 1984; Katada, 1985). These values may suggest one-to-one interaction between m-NDP kinase and the adenylate cyclase system, although an approximate turnover number of the m-NDP kinase (300,000 min^{-1}) is much greater than that of GTPase (0.8–1.7 min^{-1}) born by the G_s (Brandt and Ross, 1985; Brandt and Ross, 1986). Based on these considerations, we have attempted to detect such direct interaction between the m-NDP kinase and the component(s) of adenylate cyclase and, quite recently, found that the m-NDP kinase and the G_s can be extracted as a complexed form from membranes by detergent. Moreover, the complex formation was under the regulation of hormones and guanine nucleotides (Kimura and Shimada, 1988). These observations obviously strengthen the idea of the membrane-associated form of NDP kinase and show that the m-NDP kinase does have a direct interaction with the adenylate cyclase system. Now, we have the pure enzyme, and this will allow us to pursue studies on its interaction with the adenylate cyclase system at the molecular level.

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SUPPLEMENTARY MATERIAL

TO

MEMBRANE-ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE FROM RAT LIVER:

PURIFICATION, CHARACTERIZATION, AND COMPARISON WITH CYTOSOLIC ENZYME

Narimichi Kimura, and Nobuko Shimada

MATERIALS AND METHODS

Preparation of purified plasma membranes The purified plasma membranes from rat liver as a source of mNDP kinase were prepared essentially according to the method previously described(Kimura and Nagata, 1977) except that sucrose density gradient centrifugation was performed once in an SW 27 rotor, followed by three repeated washings by sedimentation in order to minimize carry-over of s-NDP kinase.

Purification of m-NDP kinase Purified membranes from 200 to 300 rats were pooled and resuspended in a buffer containing 10mM Tris/HCl, 2mM $MgCl_2$, 1mM EDTA, 1mM dithiothreitol(DTT), and 0.25mM PMSF, pH 7.4(TMEDP), and 100mM CHAPS was added to the suspension to a final concentration of 10mM. After membranes were solubilized for 60min at 0°C with stirring, insoluble materials were removed by centrifugation at 105,000xg for 60min. The supernatant was added to 100ml of packed Blue Sepharose CL-6B which had been equilibrated with TMEDP-1mM CHAPS and the mixture was allowed to stand for 1h with gentle shaking so that more than 95% of the mNDP kinase activity was adsorbed to the resin. The resin was washed once with the equilibration buffer and packed into a column(2.6 x 20cm). The column was washed with one column volume of the equilibration buffer, one column volume of TMEDP-1mM CHAPS containing 0.5M NaCl, and the mNDP kinase activity was eluted by TMEDP-1mM CHAPS containing 0.5M NaCl, 1M Tris/HCl and 2mM ATP(pH 7.4). The fractions which contained the enzyme activity were pooled, dialyzed and then applied on a DEAE-Sepharose CL-6B column(1.6 x 40cm) at a flow rate of 37ml/h. The column was washed with TMEDP-1mM CHAPS and the enzyme was eluted by the same buffer containing 0.1M NaCl. The pooled fractions containing enzyme activity were concentrated and then passed through an Ultrogel AcA 34 column(1.6 x 80cm) equilibrated with TMEDP-1mM CHAPS containing 0.1M KCl at a flow rate of 6.4ml/h. The peak fractions were collected and dialyzed against 10mM potassium phosphate buffer, pH 7.2 containing 1mM DTT, 0.25mM PMSF, and 1mM CHAPS and then applied on a Bio-Gel HTP column(1.6 x 15cm), followed by elution with a linear gradient consisting of 100ml each of 10 and 400mM potassium phosphate buffer, pH 7.2 containing 1mM DTT, 0.25mM PMSF, and 1mM CHAPS. The fractions containing enzyme activity were collected and concentrated on a YM-10 membrane, and then further purified by a second AcA 34 column(1.6 x 80cm). The peak fractions collected were concentrated and stored at -70°C.

Purification of s-NDP kinase The livers, weighing 200-250g, from Wistar male rats were minced and homogenized in TMEDP with the aid of a Brinkman polytron. The homogenate was centrifuged at 12,000xg for 15 min, and the resulting supernatant was further centrifuged at 105,000xg for 60 min. The ammonium sulfate precipitate of the supernatant occurred between 45 and 70% saturation was collected. The precipitate was dissolved, dialyzed against TMEDP, and then applied to a column of Blue Sepharose CL-6B(1.6x25 cm) previously equilibrated with TMEDP at the flow rate of 40ml/h. The column was washed with the equilibration buffer and then with the same buffer containing 0.3M NaCl. The enzyme was eluted with TMEDP containing 0.3M NaCl and 2mM ATP. After dialysis the enzyme was further purified by column chromatographies on DEAE-Sepharose CL-6B and Ultrogel AcA 34. The final s-NDP kinase preparation displayed a purification factor of approximately 1,700 with a specific activity of 2,500-3,500 μmol/min/mg and frequently comprises a doublet of protein bands, an apparent molecular weight of the major band being 18,300(see Fig. 2). The recovery of the s-NDP kinase activity was about 14%.

Enzyme assay Unless otherwise mentioned, both NDP kinases were assayed using the pyruvate kinase-lactate dehydrogenase coupled enzyme system(Agarwal & Parks, 1971). When kinetic parameters of these enzymes were determined, isotopic method was employed(Kimura & Shimada, 1983).

Electrophoretic methods SDS-PAGE was performed as described by Laemmli(1970), and the gels were then silver stained using Silver Stain KANTO(Kanto Chemical Co., Tokyo) after fixation by trifluoroacetic acid, or subjected to autoradiography or immunoelectrophoretic blotting.

Nondenaturing isoelectric focusing was performed at 200V for 4-5h at 4°C in cylindrical polyacrylamide gels(0.5x8 cm) prepared with 5% acrylamide, 0.25% N, N'-methylene bis(acrylamide), 10% glycerol, 1mM DTT, 1mM CHAPS, 2% Ampholines(pH 3.5-10), 0.02% ammonium persulfate and 0.001% TEMED. When s-NDP kinase was examined, CHAPS was excluded. The pH gradient was determined either by extraction with degassed water followed by direct measurement, or with pI marker proteins(Pharmacia). When enzyme activity was determined, the gels were cut into 2.5-mm pieces and the activity was extracted by homogenization followed by incubation at 4°C for 10h.

Sucrose density gradient centrifugation Linear sucrose density gradients(5-20%, 4.8ml) were prepared with TMEDP containing 0.1M NaCl and 0.1% Lubrol PX in H_2O or D_2O. A solution(200μl) containing the purified m-NDP kinase along with protein standards(fumarase, 9.1S; lactic dehydrogenase, 7.4S; and cytochrome c, 1.8S) was overlayed and the tubes were centrifuged at 43,000rpm for 14h(H_2O) or 18h(D_2O) in a Beckman SW 50.1 rotor. The standard proteins were assayed as described by Clarke(1975). Cytochrome c was measured by its absorbance at 410 nm. The s-NDP kinase was similarly examined in sucrose gradients devoid of Lubrol PX prepared in H_2O.

Analytical gel filtration Gel filtration of s-NDP kinase was performed on a Ultrogel AcA 34 column(2.6x45.5 cm) at a flow rate of 17ml/h. The Stokes radius for the enzyme was obtained from a standard curve of $(-\log K_{av})^{1/2}$ versus Stokes radius with the following protein standards: ferritin(Stokes radius, a=6.1 nm), aldolase(a=4.8 nm), bovine serum albumin(a=3.6 nm), ovalbumin(a=3.1 nm), chymotrypsinogen(a=2.1 nm), and RNase A(a=1.6 nm).

Amino acid analysis Samples were hydrolyzed in 6N HCl at 110°C for 24, 48, and 72h and analyzed with a Hitachi 835 amino acid analyzer. Values given in the table are corrected for the partial destruction of aerine and threonine by extraporation to zero hydrolysis time or for incomplete liberation of valine and isoleucine after 24h hydrolysis. Cysteine was measured as cysteic acid by performic acid oxidation. Tryptophan was measured after hydrolysis in 4N methanesulfonic acid at 110°C for 24h.

Radioiodination of m- and s-NDP kinases The m- and s-NDP kinases were radioiodinated by chloramine T method(Hunter and Greenwood, 1962). Incubation mixture contained 50 ng of each enzyme, 0.5 mCi $Na^{125}I$, and 220mM sodium phosphate, pH 7.2 in 85μl. Incubation was started by adding 15μl of 5mg/ml of chloramine T, carried out for 3 min at 0°C, and stopped by the addition of 10μl of 5mg/ml of sodium metabisulfite, followed by 10μl of 10% KI and 10μl of 2% BSA solutions. Enzymes were precipitated by the addition of trichloroacetic acid solution. The precipitates collected by centrifugation were resuspended in 20% trichloroacetic acid and centrifuged again to remove unreacted ^{125}I. The resulting pellets were rinsed with ethylether, dissolved in Laemmli's sample buffer, and then subjected to SDS-PAGE. After electrophoresis was completed, the labelled enzymes separated were extracted from gel and used for peptide mapping study.

Preparation and purification of antibodies against s-NDP kinase New Zealand White rabbits were injected intradermally with purified s-NDP kinase, initially 0.25mg/animal in complete Freund's adjuvant, followed by four times of 0.5mg of booster injections at 2 weeks intervals. Animals were bled one week after the last booster injection and heat-inactivated sera were used. Monospecific antibodies were isolated by affinity chromatography. An affinity column with the purified s-NDP kinase as the ligand was prepared by coupling 5mg of the enzyme to 1ml Affi-Gel 15 according to the manufacturer's recommendations. Antiserum(0.5-1.0ml) was applied directly on the affinity column, the column was washed with 10mM sodium phosphate, pH 7.4, containing 0.15M NaCl(PBS) and then the bound IgG was eluted from the column with 0.1M glycine hydrochloride buffer, pH 2.5. In some experiments purified IgG fractions prepared from nonimmune rabbit serum by Affi-Gel Protein A column chromatography were used instead of the serum.

Enzyme linked immunosorbent assay Enzyme linked immunosorbent assay(ELISA) was carried out as follows. Microtiter plates(96wells) were coated with the purified s-NDP kinase(50μl/well of 20μg/ml solution) for 15h at 4°C. The plates were washed three times with 0.05% Tween 20-PBS, incubated with 50μl of diluted antisera for 1h at 37°C, and then washed 2 times with 0.05% Tween 20, 2% bovine serum albumin(BSA) in PBS. After the plates were incubated with 100μl/well of a 1/500 dilution of horseradish peroxidase-conjugated goat antirabbit immunoglobin(Miles-Yeda Ltd) for 30min at 37°C, followed by washing 3 times with 0.05% Tween 20-PBS, 150μl of 0.07% 5-aminosalicylic acid-0.005% H_2O_2(pH 5.0) was added and color was developed at room temperature for 1h in the dark. After the addition of 50μl/well of 1N NaOH, the difference in absorbance measured at 492 and 610 nm was read in an ELISA reader(Corona Electric, MTP-22).

Immunoelectrophoretic blotting The purified m- and s-NDP kinases, crude rat liver extract, and purified rat liver plasma membranes were subjected to electrophoresis on SDS-acrylamide gels and then electrophoretically transferred to nitrocellulose membranes(Towbin et al., 1979) in transfer buffer of 25mM Tris, 192mM glycine, 20% methanol containing 0.005% SDS at a constant current(500mA) for 1h at 0°C. The nitrocellulose blots were incubated in TBS-3% BSA for 15min, followed by incubation with nonimmune serum, purified nonimmune IgG, antiserum or affinity-purified antibodies diluted in TBS-3% BSA for 1h at room temperature. The enzyme-antibody complexes were detected by either immunostaining or autoradiography using ^{125}I-Protein A. For staining, the nitrocellulose membranes were washed three times with TBS for 5min, once with TBS-3% BSA for 15min, and then incubated with horseradish peroxidase-conjugated goat antirabbit IgG diluted 1/400 in TBS-3% BSA for 1h at room temperature. Then the wash procedure was repeated and the color reaction was performed using a Konishiroku immunostaining kit(Konishiroku Photoindustrial Co., Tokyo). For autoradiographic method, the washed and blocked nitrocellulose membranes were reacted with ^{125}I-Protein A diluted in TBS-3% BSA(0.1μCi/ml) for 1h at room temperature. The membranes were repeatedly washed, dried, and then subjected to autoradiography.

Miscellaneous Protein concentration was determined as described by Lowry et al(1951). using bovine serum albumin as a standard.

The 105,000xg supernatant fraction of the rat liver was used as a crude extract.

The partial specific volume(v) of NDP kinase was either estimated from the data on the sucrose density gradient centrifugation experiments(m-NDP kinase) or calculated from the amino acid composition according to the procedure described by Cohn and Edsall(1943)(s-NDP kinase).

Autoradiography was performed using Kodak XAR film with intensifying screens for varying periods of time.

Materials [2,^{3}H]ADP, [8-^{3}H]GDP, [5-^{3}H]UDP, [5-^{3}H]CDP, [^{125}I]Protein A were purchased from Amersham. [^{125}I]NaI was from New England Nuclear. Blue Sepharose CL-6B, and DEAE Sepharose CL-6B were obtained from Pharmacia. Ultrogel AcA 34, and Ampholines were from LKB. YM-10 membranes were from Amicon. Affi-Gel 15, Affi-Gel Protein A, and Bio-Gel HTP were obtained from Bio-Rad. Pyruvate kinase, and fumarase were from Boehringer. Staphylococcus aureus V8 protease was purchased from Miles Laboratories. Lactate dehydrogenase, cytochrome c, papain, α-chymotrypsin(TLCK-treated), subtilisin BPN', PMSF, ATP, ADP, GDP, UDP, CDP, dTDP, NADH and Lubrol PX were from Sigma. CHAPS was obtained from Wako Pure Chemicals Ind(Tokyo, Japan). Polyethyleneimine(PEI)-cellulose thin layer plates were from Merck. Methanesulfonic acid(4N, contains 0.2% 3-(2-aminoethyl)indole) was obtained from Pierce.

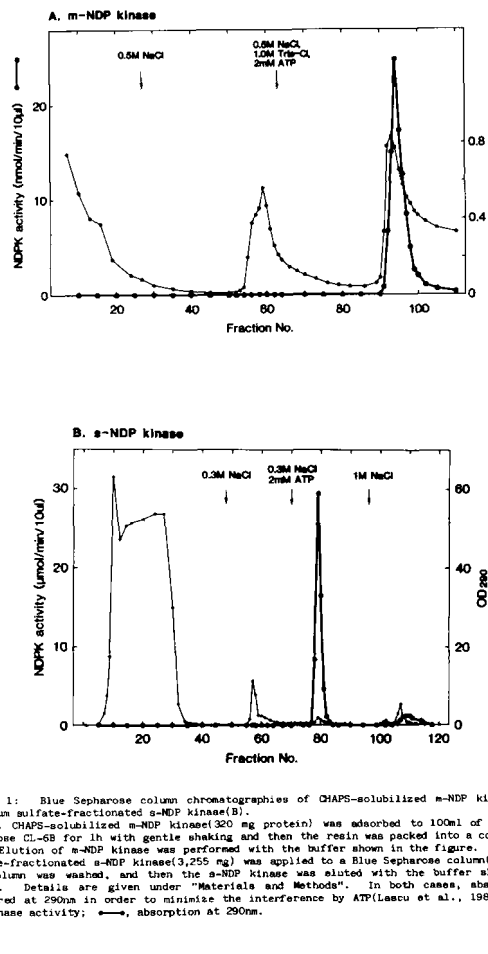

Figure 1: Blue Sepharose column chromatographies of CHAPS-solubilized m-NDP kinase(A) and ammonium sulfate-fractionated s-NDP kinase(B).

A. CHAPS-solubilized m-NDP kinase(320 mg protein) was adsorbed to 100ml of packed Blue Sepharose CL-6B for 1h with gentle shaking and then the resin was packed into a column(2.6x20 cm). Elution of m-NDP kinase was performed with the buffer shown in the figure. B. Ammonium sulfate-fractionated s-NDP kinase(3,255 mg) was applied to a Blue Sepharose column(1.6x25 cm). The column was washed, and then the s-NDP kinase was eluted with the buffer shown in the figure. Details are given under "Materials and Methods". In both cases, absorption was monitored at 290nm in order to minimize the interference by ATP(Lascu et al., 1981). ●—●, NDP kinase activity; ○—○, absorption at 290nm.

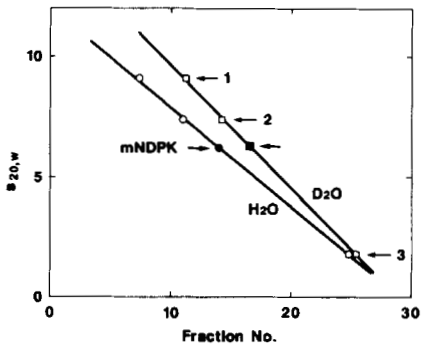

Figure 3: Sucrose density gradient centrifugation of the purified m-NDP kinase.
Samples(m-NDP kinase, 100 ng each) together with protein standards were layered onto 5-20% sucrose gradients prepared in H_2O and D_2O, centrifuged and fractionated as described under "Materials and Methods". Numbers 1-3 in the figure indicate protein standards, fumarase(9.1S), lactic dehydrogenase(7.4S) and cytochrome c(1.8S), respectively. Closed symbols(● , ■) indicate the position of m-NDP kinase.

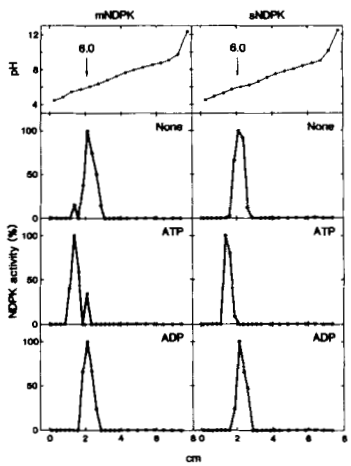

Figure 4: Isoelectric focusing of the purified m- and s-NDP kinases.
The purified m-(40 ng) and s-(5 µg)NDP kinases were preincubated with or without 1mM ATP or ADP in TMEDP for 30min at 0°C and then directly subjected to nondenaturing isoelectric focusing. The enzyme activity and pH gradient were determined as described under "Materials and Methods". Results are expressed on the ordinate as a percent of the maximal activity obtained in each gel.

Table 3

AMINO ACID COMPOSITION OF PURIFIED s-NDP KINASE

Amino acid	Residue percent	Residues / Monomer(18,300) [a]
aspartic acid [b]	8.50	14
threonine [b]	2.87	5
serine [b]	4.91	8
glutamic acid	12.20	20
proline	4.86	8
glycine	9.70	16
alanine	5.49	9
cysteine [c]	0.47	1
valine [d]	8.07	16
methionine	2.41	4
isoleucine [d]	6.56	11
leucine	7.69	12
tyrosine	2.62	4
phenylalanine	5.30	9
lysine	7.31	12
histidine	2.69	4
tryptophan [e]	1.78	3
arginine	6.58	11
total		167
molecular weight(monomer)		18,900

a) The values were obtained based on the assumption that the s-NDP kinase consists of homologous subunits.
b) obtained by extrapolation to zero hydrolysis time.
c) determined as cysteic acid after performic acid oxidation.
d) obtained after 72h hydrolysis.
e) obtained by hydrolysis in methanesulfonic acid.

Table 4

KINETIC PROPERTIES OF PURIFIED m- AND s-NDP KINASES

Substrate	Apparent Km	
	m-NDPK	s-NDPK
	mM	
ATP	1.66	1.33
ADP	0.073	0.042
GDP	0.036	0.031
UDP	0.16	0.19
CDP	0.69	0.54

The enzyme activity was determined using each radiolabeled nucleoside diphosphate listed together with 1mM ATP, or [^{3}H]GDP and varying amount of unlabeled ATP with a constant ATP/$MgCl_2$ ratio(1:5) as substrates. The radiolabeled corresponding nucleoside triphosphates formed were separated by thin layer chromatography on PEI-cellulose plates with 2N HCOOH-1.3M LiCl(1:1) as a solvent.