Complete recovery of the phosphoenzyme-forming activity of nucleoside-diphosphate kinases after reconstitution of their subunits

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Two distinct subunits [α-subunit (M_r 21 000, p*I* 7.6) and β-subunit (M_r 19 000, p*I* 6.5)] of nucleoside-diphosphate (NDP) kinases highly purified from HeLa S3 cells can be separated by FPLC using a Mono P column in the presence of 6 M urea and 1% pharmalyte (pH range between 5.0 and 8.0). Comparatively high [^{32}P]-phosphate incorporation was detected when these two subunit fractions were reconstituted in vitro. Available evidence suggests that these two enzyme subunits are necessary for the formation of phosphoenzyme, which functions as an intermediate in NDP kinase action

Nucleoside-diphosphate kinase *Enzyme subunit* *Reconstitution* *Phosphoenzyme formation* *(HeLa S3 cell)*

1. INTRODUCTION

Recently, we reported that NDP kinase (EC 2.7.4.6), which catalyzes a phosphate transfer in a wide variety of nucleoside 5′-di- and triphosphates [1], may play an important role in the initiation of cell proliferation and cell differentiation because treatment of mouse NK cells with recombinant human interleukin 2 (rIL-2, T cell growth factor) [2,3] or HeLa S3 cells with human type interferons (HuIFNs) [4] results in the rapid induction of the enzyme. Since the enzyme forms [^{32}P]phosphoenzyme, an enzyme-bound high-energy phosphate intermediate, when incubated with [γ-^{32}P]ATP in the presence of divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}), the nitrocellulose membrane method is extremely useful for purification of the enzyme [2–6]. Using this method, we purified NDP kinases to a high degree from various mammalian cells, such as mouse NK [3], Ehrlich ascites tumor [5] and HeLa S3 cells [4,6]. We found that the purified enzymes from different mammalian cells consist of two distinct subunits [α-subunit (M_r 20000–22000) and β-subunit (M_r 18000–19000)] [2–6]. However, the physiological role of each subunit in its enzyme action remains to be elucidated.

The present study has been undertaken to determine the physiological role and some biochemical properties of the enzyme subunits. Reconstitution experiments in vitro after separation of the enzyme subunits by FPLC using a Mono P column showed that it is their [^{32}P]phosphate incorporating activity that makes these two enzyme subunits necessary.

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Abbreviations: NDP kinase, nucleoside-diphosphate kinase; DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (20.0 Ci/mmol) was obtained from

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Amersham, nucleoside 5′-diphosphates (ADP, GDP, CDP and UDP), nucleoside 5′-triphosphates (ATP, GTP, CTP and UTP), dithiothreitol (DTT) and phenylmethylsulphonyl fluoride (PMSF) from Sigma, Sephacryl S200 and pharmalyte (pH range between 5.0 and 8.0) from Pharmacia, DEAE-cellulose from Whatman, hydroxyapatite (type HTP) from Bio-Rad and nitrocellulose membrane filter (type TM-1, 0.65 μm) from Toyo Roshi (Tokyo).

2.2. *Crude NDP kinase purification from HeLa S3 cells*

HeLa S3 cells (approx. 2×10^9 cells) were gently homogenized with a glass homogenizer in 1.5 ml of 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 0.5 mM PMSF, 50 mM KCl and 10% glycerol, then centrifuged for 20 min at 30000 rpm at 4°C. The supernatant obtained was concentrated with solid ammonium sulfate (65% saturation). The precipitate was dissolved in 2.5 ml of the extracting buffer, then dialyzed against the same buffer. The dialysate was used for this study as a crude NDP kinase preparation.

2.3. *Assay for phosphoenzyme formation of NDP kinase*

As we previously reported [2–5], NDP kinases in the crude and purified fractions formed [^{32}P]phosphoenzymes when incubated with [γ-^{32}P]ATP in the presence of a divalent cation (1 mM Ca^{2+} or 1 mM Mg^{2+}). The [^{32}P]phosphoenzyme formed was quantitatively determined by the nitrocellulose membrane method as described in [2–5].

2.4. *SDS-PAGE and autoradiography*

SDS-PAGE of the crude and partially purified NDP kinases was performed according to a modification of the method described by Laemmli [7], and the ^{32}P-labeled NDP kinase polypeptides were detected by autoradiography after SDS-PAGE as reported [2–6].

3. RESULTS AND DISCUSSION

NDP kinase was partially purified from the crude enzyme preparation of HeLa S3 cells by Sephacryl S200 gel filtration in the presence and absence of 7 M urea as described in the aspect of the enzyme purification from mouse NK cells [3,4]. The S200 fraction was further purified by DEAE-cellulose and hydroxyapatite column chromatographies, successively. Two distinct enzymes (F-I, the fraction passing through the column and F-II, the 0.09–0.16 M KCl fraction) could be separated by DEAE-cellulose column chromatography as in [6]. After hydroxyapatite column chromatography, these two enzyme fractions (F-I, F-II) were purified by 5–25% (v/v) glycerol density gradient centrifugation. Enzymes F-I and F-II were purified from the crude enzyme preparation (approx. 1200- and 800-fold) with an activity yield of 34 and 17%, respectively. These two purified NDP kinases (F-I, F-II) were stable in buffer A [20 mM Tris-HCl (pH 7.5) containing 0.5 M KCl, 1 mM DTT and 10% glycerol] (90% of the activities were retained after storage at −20°C for over 3 months). In addition, sedimentation analysis showed the M_r of the purified NDP kinases (F-I, F-II) from HeLa S3 cells to be approx. 80000, estimated from the marker proteins (fig.1).

To confirm the composition of the enzyme subunits of the purified NDP kinases (F-I, F-II), these two purified enzymes were separately analyzed by SDS-PAGE followed by autoradiography after incubation for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}. As shown in fig.2, two distinct [^{32}P]phosphorylated polypeptides of M_r 21000 and 19000 were detected. The molecular sizes of the [^{32}P]phosphorylated polypeptides exactly corresponded to those determined for NDP kinase subunits in the crude cell extract from HeLa S3 cells [4]. However, it should be noted that the α-subunit (M_r 21000) phosphorylation of F-I was slightly lower than that of the β-subunit (M_r 19000), whereas the α-subunit phosphorylation of F-II was slightly higher than that of the β-subunit. Similar differential phosphorylation of the enzyme subunits was observed when they were incubated with [γ-^{32}P]ATP in the presence of 1 mM Mg^{2+} instead of 1 mM Ca^{2+}. In addition, two-dimensional gel electrophoresis showed no detection of any other phosphorylating polypeptides with similar molecular sizes (M_r 18000–22000) as previously reported for the detection of NDP kinase subunits in HeLa S3 cells [4]. Both α- and β-subunits are catalytic subunits, since (i) both subunits could be

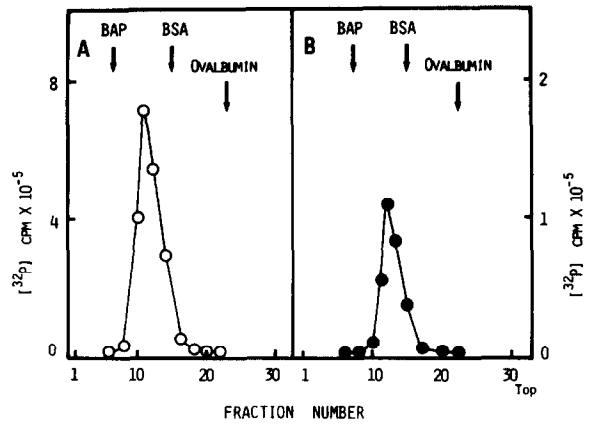

Fig.1. Glycerol density gradient sedimentation analysis of the hydroxyapatite fractions of F-I and F-II. The dialyzed and concentrated hydroxyapatite fractions of F-I and F-II (0.2 ml each) were layered separately on 4.8 ml of 5–25% (v/v) glycerol density gradients containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.1 mM EDTA and 0.5 M KCl. After centrifugation (178900 × *g* for 14 h at 4°C), the gradients were collected as 10-drop fractions from the bottom of the tube. Aliquots (5 μl) of the indicated fractions were assayed for the [^{32}P]phosphate incorporating (phosphoenzyme forming) activity as reported [2–5]. The [^{32}P]phosphate incorporating active fractions of F-I (○) and F-II (●) in the gradients were pooled separately. These two enzyme fractions (F-I and F-II) were used through this study as purified NDP kinase fractions. Arrows indicate bacterial alkaline phosphatase (BAP, M_r 92500), bovine serum albumin (BAS, M_r 67000) and ovalbumin (M_r 45000).

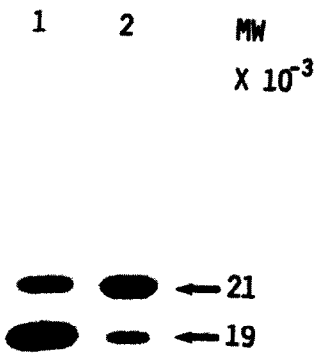

Fig.2. Autoradiogram of the [^{32}P]phosphorylated enzyme subunits after SDS-PAGE. F-I and F-II fractions (5 μg protein each) were separately incubated for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}, then immediately analyzed by SDS-PAGE following autoradiography. F-I (lane 1) and F-II (lane 2).

phosphorylated when incubated with one of the [γ-^{32}P]nucleoside 5′-triphosphates in the presence of divalent cations (Ca^{2+} and Mg^{2+}), and (ii) the [^{32}P]phosphates could be completely released from both [^{32}P]phosphorylated subunits by incubation with one of the cold nucleoside 5′-diphosphates, such as ADP, GDP, CDP and UDP, in the presence of divalent cations, resulting in [^{32}P]nucleoside 5′-triphosphates.

To separate the enzyme subunits, the purified NDP kinase (F-I) was applied on a Mono P column, previously equilibrated with polybuffer containing 6 M urea and 1% pharmalyte. After elution of the column, a pH linear gradient between pH 5.0 and 8.0 was obtained. Four distinct protein peaks (P-I to P-IV) were observed (fig.3). After dialysis against buffer A to remove urea and

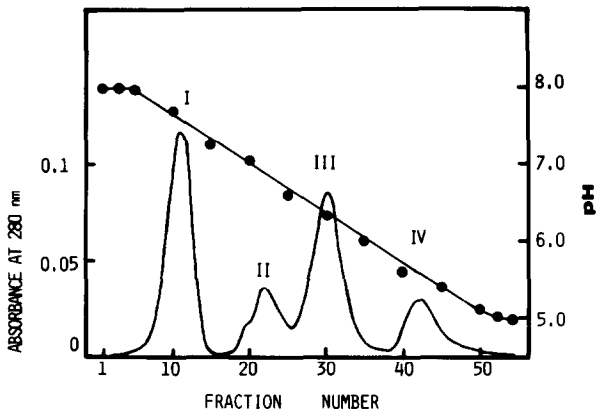

Fig.3. Subunit separation of NDP kinase I (F-I) by FPLC using a Mono P column. F-I fraction (approx. 1.5 mg protein) was applied on a Mono P column, previously equilibrated with polybuffer containing 6 M urea and 1% pharmalyte (pH range between 5.0 and 8.0). After elution of the column, a linear pH gradient between pH 8.0 and 5.0 was obtained. Distinct protein peak fractions (P-I to P-IV) were separately pooled and immediately dialyzed against buffer A [20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 0.5 M KCl and 10% glycerol] containing 0.2 M EDTA.

pharmalyte, [^{32}P]phosphate incorporation into the fractions was determined by the nitrocellulose membrane method. We concluded that P-I and P-III are NDP kinase subunits from the following experimental results: (i) the purified NDP kinase I (F-I), which contained mainly polypeptides of M_r 21000 (α-subunit) and M_r 19000 (β-subunit), was used for FPLC analysis; (ii) the pI values of P-I and P-III were close to those determined for the enzyme subunits by two-dimensional gel electrophoresis [4,6]; (iii) comparatively high [^{32}P]phosphate incorporation was observed when P-I and P-III were mixed (table 1); (iv) similar recovery of [^{32}P]phosphate incorporating activity was observed in the F-II enzyme after reconstitution of their subunits under the same conditions; and (v) the coupled pyruvate kinase-lactate dehydrogenase assay [8] detected NDP kinase activity (ADP formation from ATP) when P-I and P-III were mixed. However, it should be noted that comparatively low [^{32}P]phosphate incorporation was detected in the P-III fraction, but not in other fractions (table 1).

Table 1

Reconstitution of FPLC fractions to represent the [^{32}P]phosphate incorporating activity in vitro

Combination	[^{32}P]Phosphate incorporation into the fraction (cpm)
None	57
I	170
II	60
III	795
IV	42
I + II	331
I + III	5720
I + IV	126
II + III	670
II + IV	135
III + IV	281

The reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.1 M KCl and the indicated fractions (approx. 0.5 μg protein each) from FPLC analysis (P-I to P-IV, fig.2). The enzyme reaction was initiated by the addition of 20 μM [γ-^{32}P]ATP (3000 cpm/pmol) and incubated for 5 min in an ice bath. The [^{32}P]phosphate incorporation into the fractions was determined by the nitrocellulose membrane method as described in [2–5]

DEAE-cellulose column chromatography showed the existence of two distinct NDP kinases (F-I, F-II), composed of two similar distinct subunits (fig.3), in mammalian cells such as mouse NK [2,3], EAT [5] and HeLa S3 cells [4,6]. Although it would seem that the existence of two distinct NDP kinases is common in mammalian cells, there are some dissimilarities between these two enzymes: (i) these two enzyme subunits are differentially phosphorylated when incubated with [γ-^{32}P]ATP in the presence of divalent cations (Ca^{2+} and Mg^{2+}) (fig.2); (ii) the [^{32}P]phosphate incorporating (phosphoenzyme-forming) activity of the F-I enzyme is relatively stable as compared with that of the F-II enzyme after incubation for 20 min at 60°C [6]; and (iii) the biochemical properties of the enzyme-associated GTP-binding protein are clearly different in each enzyme [6]. However, the physiological role of the enzyme subunits involved in cellular metabolisms is not clear at present.

From the experimental results on the separation of the enzyme subunits and their reconstitution in vitro, there is a completely negligible possibility that the α-subunit (M_r 21000) of the enzyme is susceptible to limited proteolysis, resulting in the formation of the polypeptide of M_r 19000 (β-subunit). Early reports concerning the molecular size and subunit structure of NDP kinase indicated that most of the enzymes have molecular sizes ranging between M_r 80000 and 110000 [1]. We found that the molecular size of the highly purified NDP kinases (F-I, F-II) from HeLa S3 cells are approx. M_r 80000 (fig.1). This size is slightly smaller than those reported for NDP kinases from pig kidney membrane [9] and bovine brain [10], a homologous tetramer of 21 kDa polypeptide and a hexamer of 16.6 kDa polypeptide, respectively. In these reports, it is not unlikely that only β-subunit of the enzymes may be purified since it has comparatively low [^{32}P]phosphate incorporating (table 1) and NDP kinase (ADP formation from ATP) activities. This possibility is supported by our preliminary observations that: (i) our established method combining SDS-PAGE followed by autoradiography [3–6] detected two distinct [^{32}P]phosphorylated polypeptides (M_r 20000–21000 and M_r 18000–19000) in the crude NDP kinase fraction from bovine brain; and (ii) thermolysin, a type of protease [10], digested only the

α-subunit of NDP kinases highly purified from HeLa S3 cells, resulting in the formation of enzyme lacking the α-subunit, which formed a tetramer in buffer A. In addition, the molecular size (M_r 18000–19000) of the β-subunit of NDP kinases from HeLa S3 cells is close to that reported for microtubule-associated NDP kinase subunit (M_r 18000) from bovine brain [11]. Further analytical experiments to determine the physiological role of differential subunit phosphorylation in enzyme action, the phosphorylation sites in each subunit and the associating subunit of the guanine nucleotide-binding protein are currently underway.

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