ELSEVIER

Journal of Chromatography B, 660 (1994) 37–47

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Measurement of nucleoside diphosphate kinase–Nm23 activity by anion-exchange high-performance liquid chromatography

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First received 9 February 1994; revised manuscript received 30 March 1994

Abstract

A first-order assay to detect the activity of nucleoside diphosphate kinase (NDP-kinase; EC 2.7.4.6) was developed. In this assay, the activity of NDP-kinase is measured using various deoxy- and ribonucleotide triphosphates as phosphate donors and dADP as phosphate acceptor. The enzyme activity is determined by quantifying, after anion-exchange HPLC, the amount of newly synthesized dATP. Contrary to the most common coupled enzymic assays or isotopic assays the use of different donor–acceptor pairs is not restricted. The resolution of the procedure described is limited only by the chromatographic separation of substrate and product pairs participating in the reaction.

1. Introduction

The nucleoside diphosphate kinase (NDP-kinase; nucleoside triphosphate:nucleoside diphosphate phosphotransferase; EC 2.7.4.6) is a ubiquitous enzyme that mediates the catalytic phosphorylation of nucleotide diphosphates to their corresponding nucleotide triphosphates [1–3]. In this reaction, there is a transient formation of a high-energy phosphoenzyme which is mainly catalysed by ATP–Mg complexes [1,4]. Although ATP is the major donor of the terminal phosphate group required for the phosphorylation of nucleotide diphosphates, other purine or pyrimidine ribo- or deoxyribonucleotides can play the same function [1,5]. We have found that dGTP and other deoxyribonucleotide triphosphates can effectively transfer a phosphate group to dADP in the presence of NDP-kinase. In this regard, the direct participation of NDP-kinase in ATP-dependent transphosphorylation and activation of GDP bound to various G proteins remains controversial [6], but it is not unlikely that local transient pools of GDP/GTP can be modulated by NDP-kinase activity.

The recent sequence identity found between a putative Nm23 gene and NDP-kinase [7,8] has raised considerable interest in assessing better the activity of NDP-kinase during pathological disfunctions such as cellular metastasis. Strong

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0378-4347/94/$07.00
SSDI 0378-4347(94)00274-9

homologies between NDP-kinase and two proteins, Nm23 and Awd, which are poorly expressed in metastatic tumours such as human infiltrating ductal breast carcinomas [9,10] or during aberrant drosophila development, have been found [11,12]. In addition, changes in the transcription levels of Nm23 have been clearly associated with the metastatic phenotype of various tumour cell lines [13,14] and particularly of infiltrating ductal breast carcinomas [9,10]. To date, the elucidation of the molecular mechanism by which Nm23 suppresses tumor metastasis awaits the development of an accurate and specific assay to assess the putative NDP-kinase activity that has been ascribed to Nm23. Among the various procedures that have been developed for the measurement of NDP-kinase activity, the coupled enzymic assays and the isotopic assays are most commonly used [1,2,15,16]. The determination of the NDP-kinase activity by spectrophotometric coupling enzyme systems include the pyruvate kinase–lactate dehydrogenase [2] and the hexokinase–glucose-6-phosphate dehydrogenase methods [17]. These assays have the advantage of dynamically driving the reaction of the NDP-kinase and the substrate towards the formation of product. However, only specific substrates can be tested; for instance, to measure the activity of NDP-kinase by pyruvate kinase–lactate dehydrogenase assay, the substrate must have a low affinity towards pyruvate kinase and at the same time the triphosphate nucleotide must release a diphosphate nucleotide that is a good substrate for pyruvate kinase [1,2]. Several isotopic procedures have been developed to measure the activity of NDP-kinase [1,18]. A limiting factor of these methods is the necessity to provide an efficient purification procedure for the isolation of radiolabelled bioproducts that are made. In addition, these methods restrict the study of the effect of different nucleotides on the activity of NDP-kinase according to the availability of radiolabelled nucleotides.

In this paper, we describe a first-order assay for monitoring the activity of NDP-kinase. This assay is based on the efficient phosphate transfer from various deoxy- and ribonucleotide triphosphates to dADP catalysed by the nucleoside diphosphate kinase and can be also used to detect the activity of other enzymes, such as phosphatases or 5′-nucleotidases that can mask the activity of NDP-kinase in crude cell extracts.

2. Experimental

2.1. Purification of ribo- and deoxyribonucleotides

Solutions (23 m*M*) of inosine, dAMP, dADP, dGDP, dATP, dGTP, dCTP, GTP and CTP (Sigma, St. Louis, MO, USA) were applied to a Partisil 10 SAX analytical column (25 cm × 4.6 mm I.D.) (Whatman, Maidstone, UK) previously equilibrated with 0.4 *M* $NH_4H_2PO_4$ (pH 3.9). Samples were eluted with this isocratic system at a flow-rate of 1 ml/min and a pressure of about 1700–1800 psi using a Bio-Rad Model 2700 HPLC system and a multiple-wavelength Biodimension UV–Vis monitor. The pH of the purified material was brought to pH 7.0 with concentrated ammonia and the final concentration of each deoxy- or ribonucleotide was determined spectrophotometrically at 260 nm on a Beckman Model 25 instrument using the molar absorptivities reported by Cohen [19]. The purified deoxy- and ribonucleotides were stored in 1-ml aliquots at −20°C until further use.

2.2. Purification of NDP-kinase

Cytosolic NDP-kinase was purified from HL60 cell extracts as described elsewhere [20].

2.3. Cells

Human myeloid HL60 cells were maintained at 37°C under 5% CO_2 at a density of 10^5 cells/ml in RPMI medium (Flow Laboratories, McLean, VA, USA) supplemented with 5% (v/v) heat-inactivated foetal calf serum (Gibco, Grand Island, NY, USA), 2.0 m*M* glutamine, 5.5 m*M* dextrose and 100 U/ml of streptomycin and penicillin.

2.4. Cell extracts

An average of 450 ml of a cell suspension ($0.4 \cdot 10^6$ cells/ml) was spun at 280 *g* for 5 min at 15°C in an IEC-Centra 8R centrifuge, washed twice using non-supplemented RPMI medium and frozen at −70°C. Cell pellets were thawed at 37°C and resuspended in two volumes of phosphate buffer A [50 m*M* potassium phosphate (pH 7.0)–2 m*M* $MgCl_2$–1 m*M* phenylmethylsulfonyl fluoride–1 m*M* dithiothreitol–10% (v/v) glycerol]. Cells were subsequently homogenized in a polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada) set at 7.5 for three cycles of 10 s each, letting the samples stand on ice for 30 s between each cycle. Homogenates were spun at 17 624 *g* for 13 min at 4°C in a Beckman J-21C centrifuge and the supernatants were recovered and kept at −70°C until further use. Supernatants were extensively dialysed against phosphate buffer A before measuring NDP-kinase activity.

2.5. Protein determination

Protein content was determined after alkaline hydrolysis using a ninhydrin–hydrindantin reagent. Samples (100–200 μl) were dialysed against deionized water and 2–20 μl of each were placed in polypropylene tubes. Samples were dried for 60 min at 110°C and 150 μl of 13.5% (w/v) NaOH were added. The tubes were placed in an oven at 110°C for 90 min and subsequently neutralized with 250 μl of glacial acetic acid. The samples were then reacted with 500 μl of the following ninhydrin–hydrindantin solution: 2 g of ninhydrin and 150 mg of hydrindantin (Sigma) were dissolved in 65 ml of 2-methoxyethanol and then 35 ml of 4 *M* sodium acetate (pH 5.5) were added. The tubes were incubated at 110°C for 15 min. Before reading the absorbance of the samples at 570 nm, 2.5 ml of 5% (v/v) ethanol were added. Protein content was determined by interpolation on an absorbance curve obtained with samples of BSA (1–10 μg).

2.6. NDP-kinase activity

The activity of NDP-kinase was measured by determining the amount of a specific nucleotide triphosphate produced in the presence of different phosphate donor–acceptor coupled systems where the donor and acceptor possess a different base. For instance, one coupled donor–acceptor system consisted of dGTP as phosphate donor and dADP as phosphate acceptor and the rate of formation of dATP in the presence of NDP-kinase was measured as the final product. For simplification, in all assays dADP was used as phosphate acceptor but other deoxynucleotides can also be used in its place. Two different incubation media were prepared to detect the activity of NDP-kinase: (a) control reaction mixture containing 40 μ*M* of a nucleotide triphosphate (dGTP) used as phosphate donor and 0.5 m*M* $MgCl_2$; (b) the control reaction mixture supplemented with 200 μ*M* dADP, used as phosphate acceptor.

Briefly, 1–30 μg of protein were placed on ice and 150 μl of the reaction mixture (a) or (b) were added. After the addition of 15 μl of 1 m*M* inosine solution, the volume was adjusted to 300 μl with 0.4 *M* $NH_4H_2PO_4$ (pH 7.0) to give a final concentration of 20 μ*M* of the corresponding nucleotide triphosphate (donor), 0.25 m*M* $MgCl_2$ and 100 μ*M* of dADP (acceptor). The samples were incubated at 37°C for 10 min and then boiled at 100°C for 30 s. Each sample was filtered through a 0.45-μm disposable filter (Acro LC 3A; Gelman Sciences) and 200 μl were injected onto a Partisil SAX 10-μm analytical column (25 cm × 4.6 mm I.D.) (Whatman) connected to a precolumn (QMA anion exchanger; Millipore–Waters, Mildford, MA, USA). The column was equilibrated and eluted with 0.4 *M* $NH_4H_2PO_4$ (pH 3.9) at a flow-rate of 1 ml/min (ca. 1700–1800 psi). The detection of nucleotides was performed at 254 nm. The amount of each deoxy- or ribonucleotide was determined by integration of each peak and their concentrations were calculated using as external standard a mixture of different nucleotides at final amounts of 0.63, 2.50, 5.00, 10.00 and 20.00

nmol. Ten nanomoles of inosine was used as internal standard. For counting the radioactivity in the nucleotides eluted from the column, each peak was collected in a final volume of 0.5 ml and 5 ml of a water-soluble scintillation fluid were added (Scinti Verse; Fisher Scientific). Samples were counted for radioactivity on a Beckman LS 6800 automated counter.

3. Results

3.1. Determination of NDP-kinase activity

In order to investigate the possible role of the NDP-kinase in the metabolism of different nucleotides, we developed a method in which, in contrast to the coupled enzymic assays [2,17] or the isotopic assays [21,22], the nature of the deoxy- or ribonucleotide used as phosphate donor or acceptor is not restricted. In addition, the presence of enzymes in crude cell extracts such as phosphatases or 5′-nucleotidases, which can effectively compete against NDP-kinase for specific substrates, can also be detected. This method is limited only by the resolution of all possible substrate–product pairs in the HPLC effluent using the isocratic system described earlier (see the Experimental). Fig. 1 shows the chromatographic profile of a standard solution containing 10 nmol of eight different nucleotides which, with the exception of inosine (used as internal standard), could be released as final products from the reaction of NDP-kinase with the substrates used to detect the activity of the former enzyme in both purified preparations or crude cell extracts. Using 0.4 *M* $NH_4H_2PO_4$ (pH 3.9) as an isocratic system, the different nucleotides eluted from a Partisil 10 SAX column are well separated. As expected, based on their molar absorptivities at 260 nm, the absorbance of dCTP at 254 nm is lower than that of dATP or dGTP at equal concentrations. However, the absorbance at 254 nm of every nucleotide was proportional to its concentration.

Samples assayed for NDP-kinase activity were

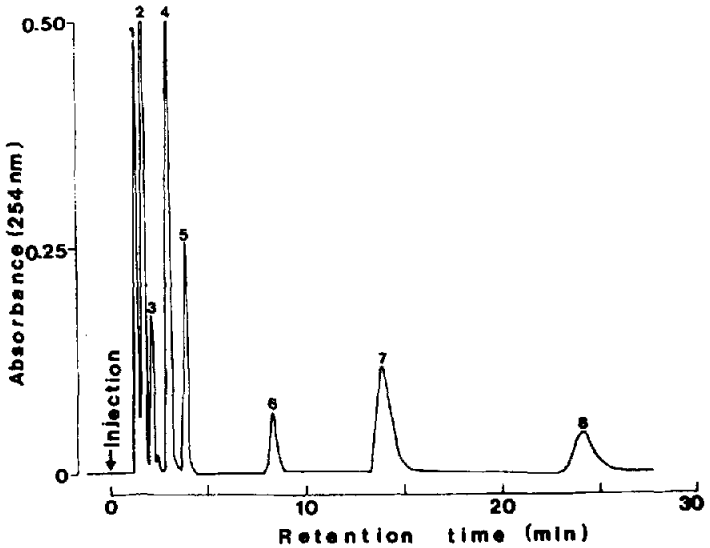

Fig. 1. Chromatographic profile of standard solution containing 10 nmol each of various nucleotides. Peaks and retention times: 1 = inosine (1.12); 2 = dAMP (1.44); 3 = dCDP (1.92); 4 = dADP (2.88); 5 = dGDP (3.68); 6 = dCTP (8.00); 7 = dATP (13.60); 8 = dGTP (23.20 min).

incubated with each of the reaction mixtures described above and the products were analysed by anion-exchange HPLC. It is worth noting that the concentrations used for both the phosphate donor (20 μM) and acceptor (100 μM) are below their K_m values, and hence the assay is of first order. In addition, the relative concentration of each product reflects the activity of the NDP-kinase under conditions in which the synthesis of a nucleotide triphosphate is promoted owing to the larger amount of the phosphate acceptor added (dADP) with respect to the phosphate donor.

3.2. Activity of cytosolic NDP-kinase isolated from HL60 cells and beef liver using dGTP as phosphate donor and dADP as phosphate acceptor

The incubation of NDP-kinase with 20 μM dGTP and a fivefold excess of dATP promoted the synthesis of dATP when 5 μg of beef liver NDP-kinase (Fig. 2A) or the same amount of NDP-kinase from HL60 cells (Fig. 2B) were used. The addition of dGTP alone led to no synthesis of dATP by either of the two NDP-kinases (see Fig. 2C and D) and dATP was not detected in the reaction mixture containing dGTP and dADP incubated at 37°C (Fig. 2E). In all instances, the same trace amounts of dAMP were also detected, and hence this corresponds to a contaminant of dATP rather than to a product released by a possible contaminant of NDP-kinase with an ATPase activity. Finally, no dATP was produced by incubating beef liver NDP-kinase (Fig. 3A) or NDP-kinase from HL60 cells (Fig. 3B) with 100 μM dADP at 37°C.

The conversion of dADP into dATP in the presence of NDP-kinase was further confirmed by incubating beef liver NDP-kinase with dGTP and dADP containing 61 pmol of [^{3}H]dADP (24.8 Ci/nmol). In this assay, a radioactive peak with a retention time equivalent to that of purified dATP was detected and was equivalent to 27 pmol of [^{3}H]dATP. No radiolabelled dATP was detected in the absence of NDP-kinase. The production of dATP by NDP-kinase was also studied using various concentrations of NDP-kinase from HL60 cells and beef liver. As shown in Table 1, using 0.1–1.2 units of the enzyme the synthesis of dATP by NDP-kinase was proportional to the hydrolysis of dGTP and also to the amount of protein used. The amount of each deoxynucleotide produced was determined using the following general equation:

$$x = K[\mathrm{N}] + i \quad (1)$$

where

$$x = \frac{\text{area under the peak of the deoxynucleotide (total absorption at 254 nm)}}{\text{area under the peak of inosine (total absorption at 254 nm)}}$$

K = slope, [N] = nucleotide concentration and i = intercept. According to Eq. 1, the concentration of nucleotide can be expressed as:

$$[\mathrm{N}] = \frac{x - i}{K} \quad (2)$$

The results presented here demonstrate that regardless of the enzyme source, the activity of NDP-kinase can be measured by determining the amount of dATP produced and released by the enzyme when dGTP is used as a phosphate donor and dADP as acceptor.

3.3. Determination of NDP-kinase activity in crude cell extracts from HL60 cells

The possible interference of phosphatases or 5′-nucleotidases in the determination of NDP-kinase activity in crude preparations was studied using cell supernatants prepared from HL60 cells and crude 5′-nucleotidase from *Crotalus adamenteus* venom. As shown in Fig. 4A, there is no dATP produced after the addition of 0.8 U of 5′-nucleotidase to a reaction mixture containing beef liver NDP-kinase (5 μg), 20 μM dGTP, 100 μM dADP and 0.25 mM $MgCl_2$. A major peak with a retention time close to those of inosine and dAMP was detected and this may correspond to an unresolved mixture of inosine,

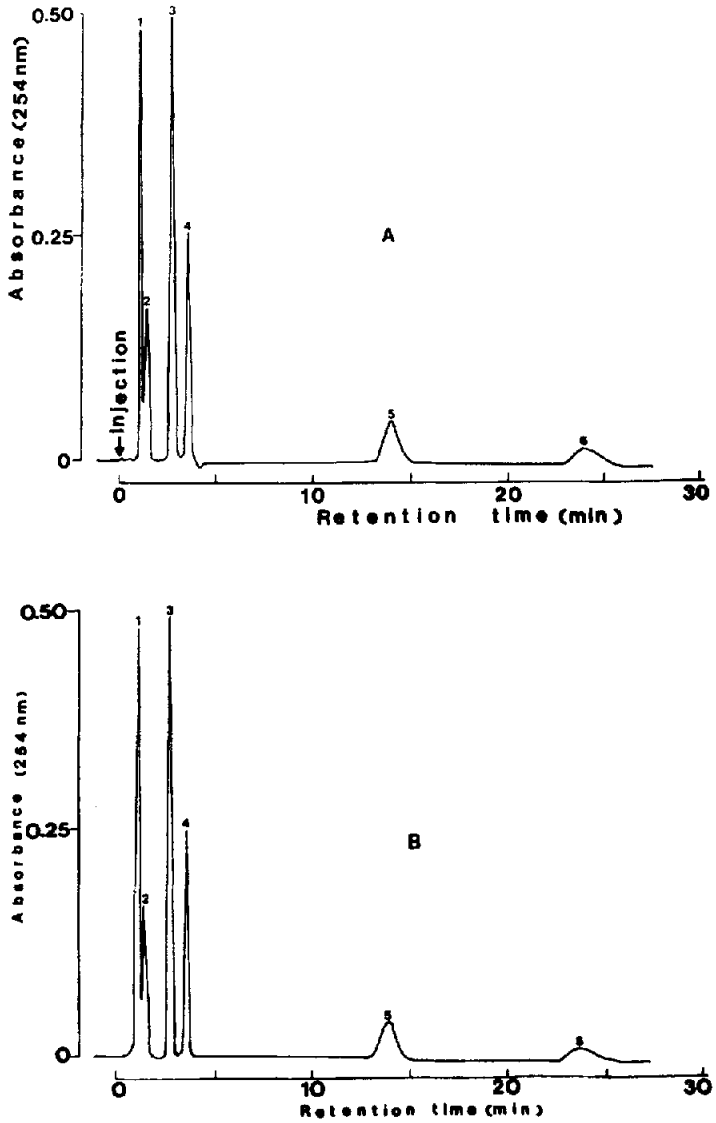

Fig. 2. Detection of NDP-kinase activity using dGTP as phosphate donor and dADP as phosphate acceptor. Peaks and mean retention times: 1 = inosine (1.13); 2 = dAMP (1.46); 3 = dADP (2.87); 4 = dGDP (3.67); 5 = dATP (13.62); 6 = dGTP (23.22 min). (A) Synthesis of dATP by beef liver NDP-kinase; (B) synthesis of dATP by NDP-kinase isolated from HL60 cells; (C) NDP-kinase from beef liver incubated with control reaction mixture containing 20 μM dGTP and 0.25 mM $MgCl_2$ in 0.4 M ammonium phosphate buffer (pH 7.0); (D) NDP-kinase from HL60 cells incubated with control reaction mixture containing 20 μM dGTP and 0.25 mM $MgCl_2$ in 0.4 M ammonium phosphate buffer (pH 7.0); (E) complete reaction mixture: 20 μM dGTP, 100 μM dADP and 0.25 mM $MgCl_2$ in 0.4 M ammonium phosphate buffer (pH 7.0), incubated at 37°C in the absence of NDP-kinase.

adenosine and guanosine produced by the phosphohydrolase activity of the 5'-nucleotidase. In contrast, as shown in Fig. 4B, dialysed supernatants prepared from HL60 cells promoted the synthesis of dATP using dGTP as phosphate donor and dADP as acceptor. We have recently

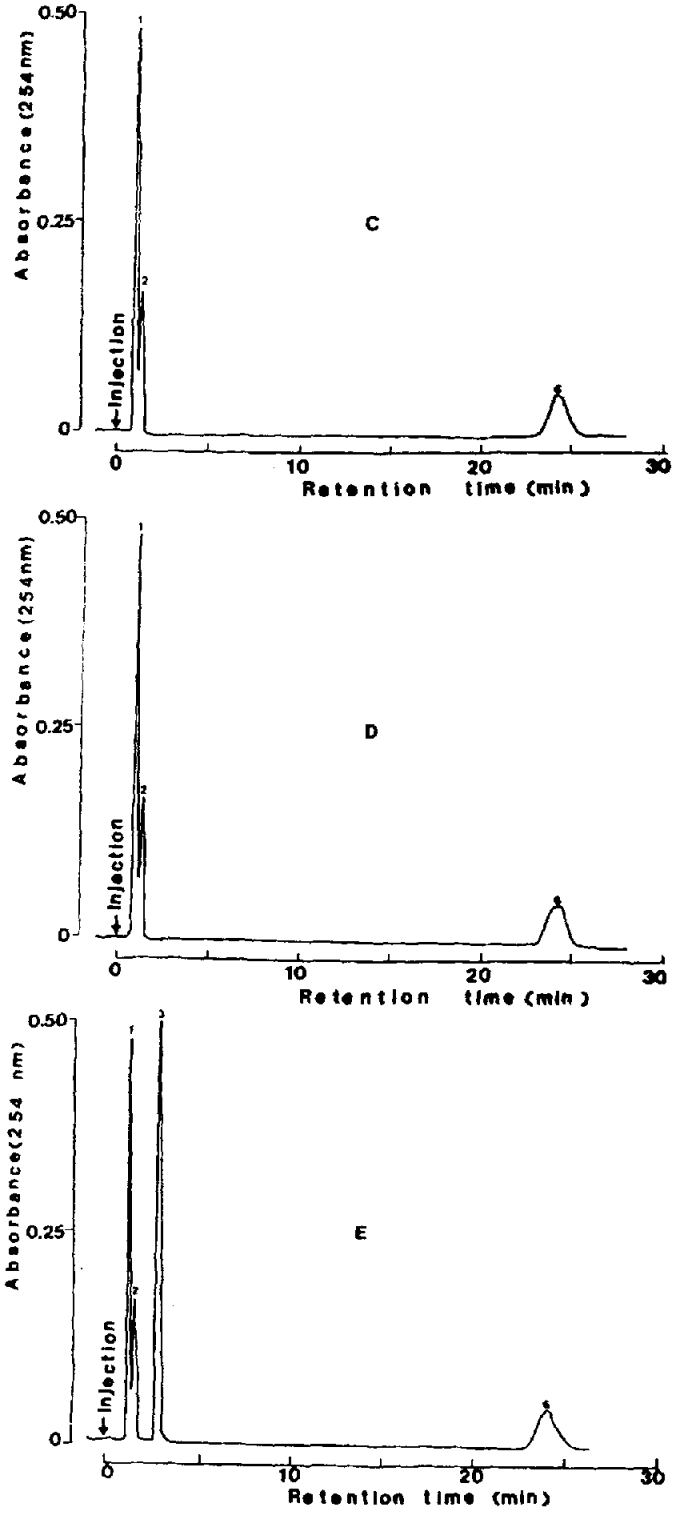

Fig. 2 *(continued).*

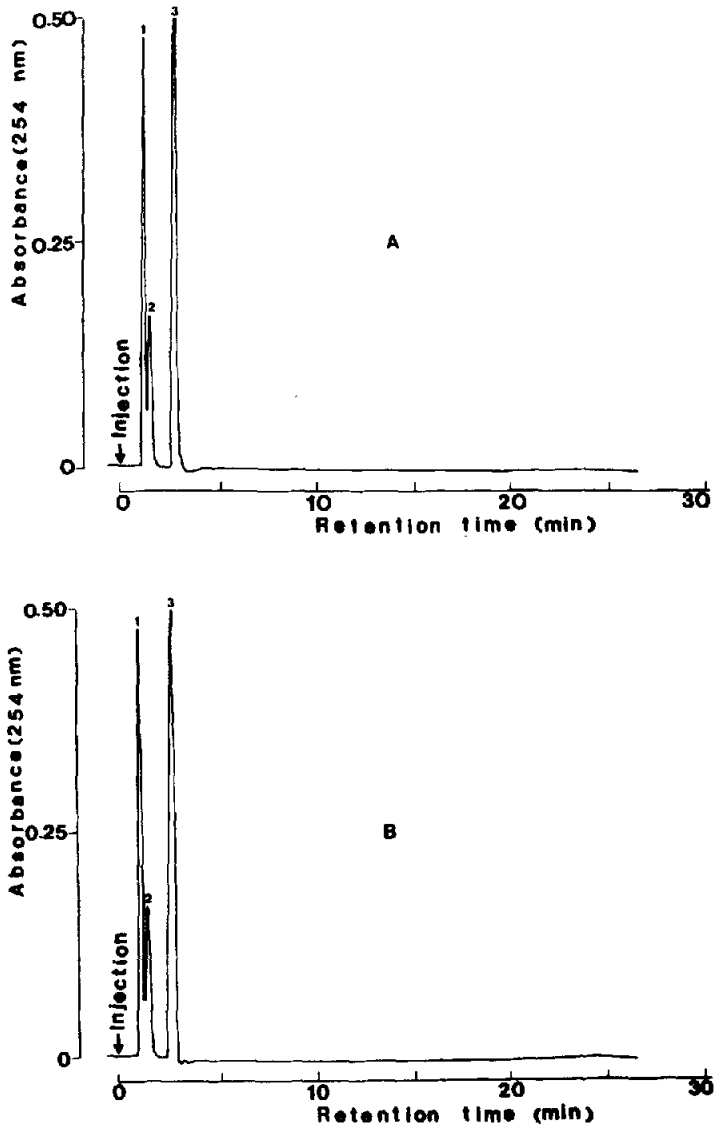

Fig. 3. Chromatographic profile of reaction mixture in the absence of dGTP as phosphate donor. Synthesis of dATP by either (A) beef liver NDP-kinase or (B) NDP-kinase from HL60 cells did not take place in the presence of dADP alone. Peaks and mean retention times: 1 = inosine (1.13); 2 = dAMP (1.46); 3 = dADP (2.87 min).

reported that the addition of anti-NDP-kinase monoclonal antibodies to HL60 cell supernatants can selectively precipitate NDP-kinase activity [20]. This in turn causes a significant decrease in the amount of dATP synthesized by the cell extracts using dGTP and dADP as a coupled donor–acceptor system [20]. Hence the synthesis of dATP by dialysed supernatants from HL60 cells is directly related to the activity of NDP-kinase and the possible presence of trace amounts of nucleotide phosphates or 5′-nucleotidases in this fraction does not interfere with

Table 1
NDP-kinase activity at different enzyme concentrations

Enzyme units	dATP produced (nmol/min)	dGTP hydrolysed (nmol/min)
0.1	0.138	0.146
0.2	0.288	0.291
0.4	0.559	0.580
0.8	1.210	1.280
1.2	1.720	1.850

the assay used to determine the activity of NDP-kinase.

3.4. Determination of K_m values for different nucleotide triphosphates using purified cytosolic NDP-kinase from HL60 cells

Although the concentrations of each nucleotide used to detect the activity of NDP-kinase were below their corresponding K_m values, this method can also be used to determine the K_m values of different nucleotide triphosphates. This was achieved by measuring the synthesis of dATP using dADP as phosphate acceptor. Increasing concentrations of each deoxy- or ribonucleotide triphosphate were added to a reaction mixture containing a fixed amount of cytosolic NDP-kinase (10 μg) purified from HL60 cells. The final concentration of dADP used in each assay as phosphate acceptor was proportionally increased in order to keep a fivefold excess of the phosphate acceptor over the phosphate donor. Table 2 shows the K_m values obtained for different nucleotide triphosphates.

4. Discussion

Among the various procedures used to determine the activity of NDP-kinase, the coupled enzymic assays, namely the pyruvate kinase–lactate dehydrogenase method [2] and the hexokinase–glucose-6-phosphate dehydrogenase assay [17], are the most extensively used. Although these methods have relative ease of operation, the major limitation encountered consists in restricting the use of specific nucleotides as enzyme substrates. In this paper, we have described a method that overcomes such restriction. This method is a first-order assay in which a phosphate donor–acceptor coupled system is used at concentrations below their corresponding K_m values and the phosphate donor and acceptor possess a different base. This facilitates both the quantification and the identification of the enzyme products. It is important to note that contrary to other standard procedures used to measure the activity of NDP-kinase, this technique allows the simultaneous quantification of substrate consumption and product formation. However, for simplification only the amount of product (dATP) was determined and this was proportional to the amount of enzyme added to a fixed concentration of substrate. The small amount of enzyme used in this assay ($\leq$30 μg), the boiling and filtration procedures used to stop the reaction and the high affinity of the remaining protein towards the Partisil 10 SAX resin allow the detection of nucleotides at 254 nm without any protein interference. This method can be used to measure the activity of pure NDP-kinase preparations or in crude cell extracts and therefore it us useful in both enzyme kinetic studies and as a routine assay to follow enzyme recoveries throughout different purification steps. In this respect, the analysis of the chromatographic profile of the products released by crude enzyme preparations can be used to detect the activity of other enzymes such as phosphatase or 5′-nucleotidases that can interfere with the measurement of NDP-kinase using other conventional methods.

The K_m values obtained for dGTP (105.4 μM); GTP (163.6 μM), dCTP (433.0 μM) and CTP (446.0 μM) using purified cytosolic NDP-kinase from HL60 cells are similar to those reported for NDP-kinase isolated from other sources using coupled enzymic assays [1,2,23]. Using the assay described here, studies dealing

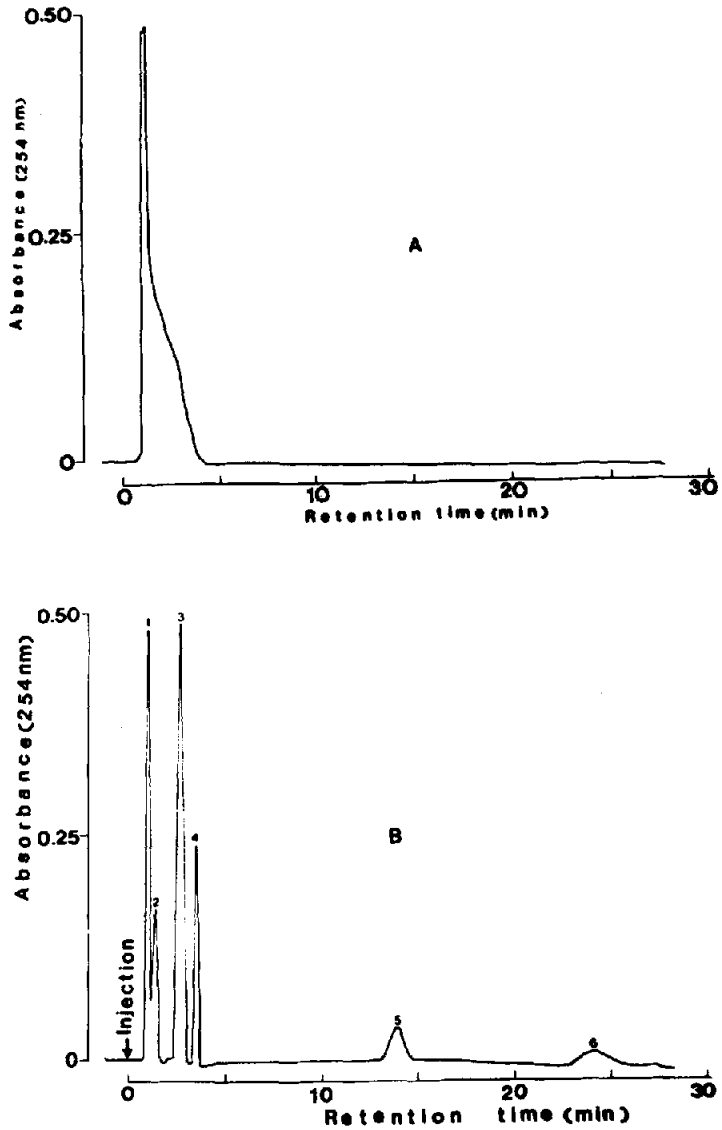

Fig. 4. Chromatographic profile of complete reaction mixture in the presence of NDP-kinase and 5'-nucleotidase. (A) Addition of 0.8 U of 5'-nucleotidase to complete reaction mixture (20 μM dGTP, 100 μM dADP, 0.25 mM $MgCl_2$ and 0.2 U of NDP-kinase) fully abrogates synthesis of dATP leading to an unresolved mixture of nucleotides with a retention time of 1.22 min (peak 1). Arrow indicates the actual injection time. (B) Detection of NDP-kinase activity in a crude cytosolic preparation of NDP-kinase from HL60 cells. Crude dialysed supernatants from HL60 cells incubated with complete reaction mixture promoted the synthesis of dATP using dGTP as phosphate donor.

with the kinetics of the NDP-kinase in the presence of different 5'-deoxy- and ribonucleotide analogues of clinical interest are in progress.

Acknowledgement

This work was supported by Health Canada and by NCI Grant no. 567 to J.-M.L.

Table 2
Ligand constants

Ligand	Correct K_m[a] (μM)	Apparent V_{max} (moles dATP/min)
dGTP	105.4	$3.1 \cdot 10^{-5}$
GTP	163.6	$4.5 \cdot 10^{-5}$
dCTP	433.0	$3.3 \cdot 10^{-4}$
CTP	446.0	$6.7 \cdot 10^{-4}$

[a] All K_m values were corrected as described in ref. [2]. Briefly, $1/V_0$ values expressed as dATP produced in nmol/min were plotted against the reciprocal of the substrate concentration and the extrapolation to the abscissa estimated by linear regression analysis [23], yielded $-(1/K_m)$.

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