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Nucleoside diphosphate kinase from human erythrocytes: purification, molecular mass and subunit structure

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A new procedure for the purification of nucleoside diphosphate kinase from human erythrocytes is described. The enzyme (105 kDa by gel filtration) is made up of two different kinds of subunits (19.0 and 20.5 kDa), both displaying enzymatic activity. The probable subunit structure of the enzyme is hexameric. The discrepancies related to earlier work are discussed.

Nucleoside diphosphate kinase; Affinity chromatography; Subunit structure; (Human erythrocyte)

1. INTRODUCTION

NDP kinase catalyses the phosphorylation of the non-adenine nucleoside diphosphates to the corresponding nucleoside triphosphates [1]. Since the enzyme is nonspecific with respect to the substrate nucleotides, it is believed to represent the link between the oxidative phosphorylation and various cell reactions requiring non-adenine nucleoside triphosphates. The association of NDP kinase with various G-proteins ([2] and refs cited therein) seems to indicate that one physiological substrate of the enzyme could be the protein-bound GDP, as suggested for tubulin [3], although the concept was criticized [4].

NDP kinase was purified from several sources to apparent homogeneity [2,3,5-11] and was shown to be a hexamer [2,3,5,8,10] or a tetramer [7,11]. The enzyme from human RBC was purified at a fairly large-scale [12,13] and some of its properties studied [13,14]. The specific activity of the purified enzyme was 100 U/mg [12] or 1000 U/mg [13] and both preparations incorporated only 3 mol of phosphate per 10^5 g of protein. Surprisingly, the subunit molecular mass and the subunit structure of the human enzyme have not yet been determined. We report below an improved purification method and the molecular mass of the native enzyme as well as of its subunits.

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Abbreviations: NDP kinase, nucleoside diphosphate kinase (EC 2.7.4.6); RBC, red blood cells; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; 8-BrIDP, 8-bromoinosine 5'-diphosphate

2. MATERIALS AND METHODS

2.1. *Chemicals*

ATP, lactate dehydrogenase and pyruvate kinase were obtained from Boehringer, Mannheim. Cibacron Red 3B-P (Ciba-Geigy) was coupled to cross-linked Sepharose 6B as described in [15] for Cibacron Blue 3G-A. 8-BrIDP was prepared as in [16].

2.2. *Analytical procedures*

The NDP kinase activity was measured with 0.3 mM 8-BrIDP as acceptor nucleotide and 1.0 mM ATP as donor nucleotide as previously reported [6] except that the final volume was 0.52 ml and 0.5 mg/ml of BSA was included in the assay mixture. One unit of NDP kinase activity catalyses the formation of 1.0 μmol of ADP per min at 25°C. Protein was measured as in [17], using BSA as a standard. Protein was precipitated from the hemolysate with acetone containing 1% of 1 M HCl before the measurement. SDS-PAGE was performed both according to Laemmli [18] or to Weber and Osborn [19], using a 15% gel.

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For the preparative recovery of the protein bands (Laemmli's system), no comb was used and the sample was applied over the length of the concentration gel. When the tracking dye reached the bottom, the protein bands were visualized with 8-anilino-1-naphthalene sulphonate [20] and cut with a razor blade under UV light. The proteins were eluted overnight with 20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1.0 mM EDTA, 10 mM β-mercaptoethanol and precipitated with cold acetone. The pellet was washed twice with cold acetone and dissolved in 50 μl of 50 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 10 mM β-mercaptoethanol, 8.0 M urea. The enzyme activity was partially recovered by dialysis against the above buffer without urea using the set-up described by Overall [21].

2.3. *The purification procedure*

100 ml of RBC were washed twice with cold saline, lysed with 100 ml of distilled water and kept frozen until used. They were thawed in 600 ml of distilled water, the pH adjusted to 8.2–8.4 with 1 M NaOH, and gently shaken with 100 ml of carbon tetrachloride for 5 min. Cell debris and excess solvent were discarded after a 10 min centrifugation at about $1000 \times g$. The clear hemolysate was pumped through a 6×4 cm column of Cibacron Red 3B-P Sepharose at a flow rate of about 5 ml/min. Over 90% of the enzyme bound to the column. The unbound proteins were washed with 20 mM Tris-HCl (pH 7.2) containing 1 mM EDTA until the absorbance at 280 nm was 0.05 or less. NDP kinase was sharply eluted with 100 μM ATP in the above buffer. The active fractions were pooled and dialysed against a saturated solution of ammonium sulphate made in 100 mM phosphate buffer (pH 7.0), containing 1 mM EDTA. The next day the precipitate was recovered by centrifugation, resuspended in a minimum volume of ammonium sulphate solution and stored at 4°C. At this point, the enzyme was 40 to 70% pure as ascertained by the SDS-PAGE analysis. The two contaminating proteins (of about 25 and 37 kDa) were eliminated by gel filtration on Sephadex G200 SF in the presence of 6 M urea as described by Ohtsuki et al. [7]. The specific activity increased in the affinity step by over 8000-fold. The yield was 40 ± 5% in several preparations. The final specific activity under the conditions described was about 800 U/mg.

3. RESULTS AND DISCUSSION

3.1. *Purification of human erythrocyte NDP kinase*

The affinity chromatography on immobilized Cibacron Blue was very successfully used for the purification of NDP kinase from other sources [2,5,6]. Here we used Cibacron Red 3B-P Sepharose because of the better stability of the dye-matrix bond. The success of the dye-mediated affinity chromatography is due to the probable location of the dye-binding site (active site?) on or near the enzyme surface [22], which allows the direct interaction with the immobilized dye. New batches of affinity material must be tested in order to find the optimal ATP concentration to be used for the elution. Using a weakly substituted gel the non-specific, ion-exchange binding of other proteins is minimal, especially at pH 8 [23], although a larger gel-to-protein ratio has to be used. A key feature of the affinity experiment was the binding step at a pH above 8.0 (i.e. greater than the isoelectric point of hemoglobin), while the washing and the elution occurred at pH 7.2. This pH shift ensured a very rapid decrease in absorbance at 280 nm from about 70 to almost nil, and a sharp elution of NDP kinase with a low concentration of ATP. We have previously shown that pig heart NDP kinase in the absence of divalent ions bound ATP at pH 7.0 with a K_d in the low μM range [24]. The behaviour of the human enzyme in the chromatographic experiment seems to indicate the same. The efficient washing of the unbound and weakly bound protein was critical for obtaining a high purification factor. In a typical experiment, less than 0.001% of the starting hemoglobin was found in the purified enzyme.

3.2. *The molecular mass of the native NDP kinase*

By gel filtration of the native enzyme on Sephadex G200 SF the molecular mass was found to be 105 kDa (fig.1A). The half width of the NDP kinase peak was equal to that of the marker proteins, a feature which demonstrated that under our conditions no equilibrium with lower association forms was present. The inclusion in the buffer of 1 mg/ml of BSA was essential for the recovery of NDP kinase in a single, symmetrical peak. In the absence of BSA, the recovery of activity was rather low and the peak was broader. It is probable that the previously reported lower values for the molecular masses of NDP kinases (55–80 kDa) were obtained under conditions where the hexameric protein was in equilibrium with lower order oligomers.

3.3. *Subunit molecular mass of NDP kinase*

The SDS-PAGE analysis according to Laemmli [18] showed two protein bands, both having enzymatic activity upon renaturation (see section 2). By running appropriate standards, the molecular masses of the two subunits were found to be 19.0 and 20.5 kDa (fig.1B). The polypeptide purified by preparative electrophoresis and re-run under the same conditions showed the expected difference in molecular mass, with no cross-contamination. The same two band pattern was obtained by SDS-

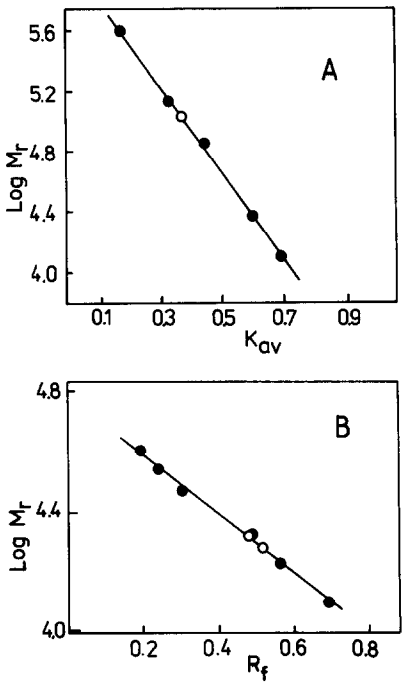

Fig.1. Determination of the molecular mass of native (A) and SDS-dissociated (B) nucleoside diphosphate kinase. (A) The log of M_r vs K_{av} in a gel filtration experiment on Sephadex G200 SF, equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 1.4 mM mercaptoethanol and 1 mg/ml BSA. The standard proteins (filled circles) were, from left to right, horse serum cholinesterase (390 kDa), beef muscle lactate dehydrogenase (140 kDa), pig heart malate dehydrogenase (70 kDa), rabbit muscle adenylate kinase (25 kDa) and horse heart cytochrome *c* (13.5 kDa). (B) Log of M_r vs R_f obtained in SDS-PAGE according to Laemmli [18]. The standard proteins (filled circles) were, from left to right, rabbit muscle aldolase (40 kDa), beef muscle lactate dehydrogenase (35 kDa), beef erythrocytes carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), horse muscle myoglobin (17 kDa) and horse heart cytochrome *c* (13.5 kDa).

PAGE analysis using the buffer system of Weber and Osborn [19]. Therefore, the difference in M_r of the two polypeptides is not an artefact of the Laemmli technique. The M_r of the two polypeptide chains are too close to allow their separation by gel filtration. However, a partial separation was obtained by gel filtration on Sephadex G200 SF in the presence of 6 M urea (not shown). Fractions eluted earlier were richer in the 20.5 kDa polypeptide, whereas fractions eluted later were richer in the 19.0 kDa polypeptide. Two protein bands with NDP kinase activity had been found in Ehrlich ascites tumor cells [11], HeLa cells [11] and rat liver [5], but only one band in the enzyme purified from other sources [2–4,6,8,9]. Since this phenomenon is observed in NDP kinase purified from RBC from single donors as well, and as the whole purification procedure takes just a few hours (which makes proteolysis improbable) we believe that the human enzyme is built up of two kinds of polypeptide chains, having slightly different M_r values. The nature of the difference between the subunits, as well as its relationship with the NDP kinase isozymes [14] is currently being investigated.

3.4. *Conclusions*

Our results suggest that NDP kinase from human RBC is a hexamer built up of two kinds of subunits. As a small protein with an essential function, NDP kinase is believed to be highly conserved during evolution. In fact, the catalytic mechanism, the high metabolic stability and the subunit M_r values are similar in all NDP kinases, from bacteria to man. The only discrepancy refers to the M_r of the native enzyme, which in turn is used for the calculation of the number of the subunits. Eventually the cross-linking experiments would give a clear answer. Unfortunately, our preliminary attempts with glutaraldehyde and dimethyl suberimidate as cross-linkers were not successful.

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