DIFFERENTIAL SENSITIVITY TO *p*-CHLOROMERCURIBENZOATE AND UREA OF SOLUBLE AND SEPHAROSE-BOUND PIG HEART NUCLEOSIDE DIPHOSPHATE KINASE

Dao MINH DUC, Ioan LASCU, Horea PORUMB*, Ofelia GOZIA**, Horst D. SCHELL** and Octavian BÂRZU†

*Department of Biochemistry, *Department of Biophysics, Medical and Pharmaceutical Institute, 3400 Cluj-Napoca, Japan* and ***Institute of Biological Sciences, 77748 Bucharest, Romania*

Received 19 February 1981

1. Introduction

Nucleoside diphosphate kinase (EC 2.7.4.6) catalyzes the reaction $N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$, where NDP and NTP stand for nucleoside di- and triphosphates, respectively. The enzyme from various species and tissues can be purified to electrophoretic homogeneity, nevertheless the structural studies are complicated by difficulties in obtaining large amounts of pure NDP kinase, and in the case of the mammalian enzyme by the presence of isoenzymes [1–5]. In view of analyzing the molecular properties of the pig heart NDP kinase, we have recently set up a technique for rapidly obtaining large amounts of enzyme by affinity chromatography on Cibacron Blue 3G-A Sepharose 4B-CL [6].

Here we report on the differential sensitivity to pCMB and urea of soluble and matrix-bound NDP kinase.

2. Materials and methods

2.1. *Chemicals*

All natural nucleotides, substrates and coupling enzymes were products of Boehringer Mannheim (a generous gift of Prof. F. H. Schmidt). CNBr-activated Sepharose 4B was supplied by Pharmacia, Uppsala. 8-BrIDP was obtained by oxidative deamination of 8-BrADP [7]. ADP was brominated to 8-BrADP in aqueous solutions essentially as in [8].

Abbreviations: NDP kinase, nucleoside diphosphate kinase; pCMB, *p*-chloromercuribenzoate; 8-BrIDP, 8-bromoinosine 5′-diphosphate; 8-BrADP, 8-bromoadenosine 5′-diphosphate

† To whom correspondence should be addressed

2.2. *Purification and immobilization of pig heart NDP kinase*

Pig heart NDP kinase (spec. act. approx. 1000 units/mg protein) was obtained as in [6]. 0.8 g of CNBr-activated Sepharose 4B was suspended in 10 ml 1 mM HCl then washed several times with the same solution on a sintered glass funnel. Thereafter the gel was washed 4 times with 20-ml portions of 0.1 M sodium borate buffer (pH 8.0) containing 0.5 M NaCl and suspended in 15 ml of the same buffer. NDP kinase (suspension in ammonium sulfate solution at 80% saturation) was dialysed against 20 mM phosphate buffer (pH 7.4) containing 2 mM EDTA, for 24 h. The specific activity of the enzyme decreased after dialysis to about 750 units/mg of protein. 400 μl dialyzed enzyme solution containing about 0.8 mg of protein were diluted to 4 ml with 0.1 M borate buffer (pH 8.0) containing 1 mM ADP, and stirred with the activated gel for 3 h at room temperature. Excess soluble protein was then removed by washing the gel alternately with cold coupling buffer and 0.1 M acetate buffer (pH 4.5) containing 1 M NaCl. 5 vols. of 1 M Tris-HCl (pH 8.0) were then added to the matrix-bound derivative and the mixture was stirred for 3 h at room temperature, to allow the complete blocking of the remaining activated groups on Sepharose. After washing with bidistilled water to remove excess Tris-HCl buffer, the gel was suspended in 20 ml 20 mM phosphate buffer (pH 7.0) and stored at 4°C.

NDP kinase could be linked to Sepharose 4B with retention of approx. 25% of the specific activity of the soluble enzyme. Since the kinetic constants for nucleotides of the matrix-bound and soluble enzymes do not differ essentially, the decrease in the specific activity might be the result of the dissociation of NDP

kinase into inactive subunits upon immobilization. 1 ml of packed gel contained 27–38 units. When ADP was absent during the immobilization, the specific activity decreased to only 5–8% of that of the soluble enzyme.

2.3. *Determination of NDP kinase activity and other analytical procedures*

NDP kinase activity was measured with a coupled assay system involving pyruvate kinase and lactate dehydrogenase [7]. When maximal rates were required 1 mM ATP and 0.2 mM 8-BrIDP were used. In routine determinations, 'suboptimal' concentrations of substrates were used, i.e. 0.3 mM ATP and 0.1 mM 8-BrIDP. The reaction medium contained in a final vol. of 1 ml and 25°C, 50 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 0.1 mM NADH, 1.5 units of each pyruvate kinase and lactate dehydrogenase, and nucleotide substrates. The reaction was usually started by addition of NDP kinase and the absorption decrease at 366 nm was measured with an Eppendorf 1101 M type photometer, equipped with a W+W 4410 type recorder (full scale deflection 0.25 absorbance unit). In the case of immobilized NDP kinase the cuvette content was continuously stirred using a mixing device* adaptable to Eppendorf photometers by the simple replacement of the cell holder [9]. Pipetting of gel suspension was found to be most accurately done using capillary micropipettes of 10 or 20 μl (Labora, Mannheim). Protein concentration was determined as in [10].

3. Results

3.1. *The kinetic properties of soluble and immobilized NDP kinase*

The immobilized NDP kinase does not differ significantly from the soluble one from the point of view of the kinetic constants. Thus, the affinities for nucleotide substrates are alike: K_m^{ATP} is 0.18 mM (soluble enzyme) and, respectively, 0.23 mM (immobilized enzyme) at fixed (0.2 mM) 8-BrIDP concentration; $K_m^{8\text{-BrIDP}}$ is 0.14 mM (soluble enzyme) and, respectively, 0.20 mM (immobilized enzyme) at fixed (1 mM) ATP concentration. Both the soluble and immobilized NDP kinase are inhibited by excess nucleoside diphosphates, but not by excess ATP, and the optimal pH ranges between 7.5–8.0 in both cases. However, while the activity of the soluble enzyme decreases rapidly towards the alkaline domain (50% of the maximal activity is at pH 8.5), the immobilized enzyme retains 65% of its maximal activity even at pH 9.0. The thermal stability of the immobilized NDP kinase is greatly enhanced; the activity is not affected by 30 min of incubation at 70°C, temperature at which the soluble enzyme (0.5 mg of protein/ml) is inactivated in 2 min.

3.2. *Effect of pCMB on soluble and immobilized NDP kinase*

NDP kinase of various origins was shown to have 1 SH group/subunit, which reacts slowly with pCMB and does not react with DTNB [3,11,12]. In line with this, the pig heart enzyme is inhibited by pCMB (85% inhibition at 10^{-4} M pCMB, in 30 min). The nucleoside diphosphates, but not the corresponding mono- and triphosphates, protect the enzyme against the inactivation, the maximum effect being obtained at 1 mM ADP or GDP (fig.1). This particular behavior is

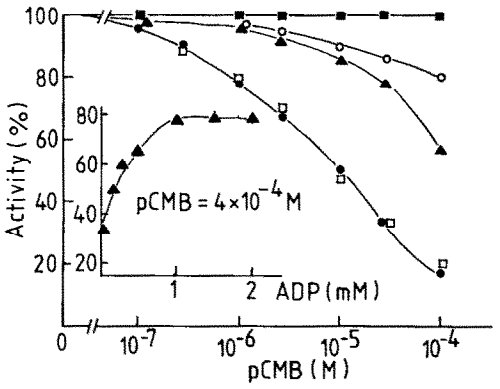

Fig.1. Effect of pCMB on soluble and Sepharose-bound pig heart NDP kinase. 20 μg of soluble NDP kinase (•) and, respectively, 350 μl of immobilized enzyme corresponding to 35 μl of packed gel (▪), were incubated at room temperature for 30 min in 1 ml 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and pCMB (concentrations between 10^{-4}–10^{-8} M). The mixture was continuously stirred to avoid the sedimentation of insoluble NDP kinase. Where indicated, the medium containing the soluble enzyme was suplemented with 1 mM ATP (▫), 1 mM ADP (▲) or 1 mM GDP (○). 2–10 μl of incubation mixture were then withdrawn for the measurement of NDP kinase activity as in section 2. Inset: protection by ADP of soluble NDP kinase against inactivation by 4 × 10^{-4} M pCMB. The activity in the absence of pCMB was considered as 100%.

* The mixing device with the thermostated cell holder is now commercially available from Eppendorf Gerätebau, Hamburg

even more intriguing as the enzymes from other sources are protected to roughly the same extent by any nucleotide, regardless the number of phosphoryl groups in the molecule, or the nature of the heterocycle. Upon immobilization, the sensitivity of NDP kinase to the inhibitory effect of pCMB is lost. The simplest explanation would be that the SH groups sensitive to pCMB attack do not belong to the active site, but are essential for maintaining the active quaternary structure of the enzyme.

3.3. *Effect of urea on soluble and immobilized NDP kinase*

The soluble enzyme is not affected by 30 min incubation with urea up to 4 M concentration. At 7 M urea, the soluble enzyme is inhibited by 60%, while the immobilized enzyme still maintains its full activity. The immobilized NDP kinase only becomes inhibited at concentrations between 8–9 M urea (fig.2). Nevertheless, the recovery of enzyme activity could not be achieved upon diluting to 6 M urea even after 30 min of incubation. It is worth mentioning the combined effect of pCMB and urea on the immobilized pig heart NDP kinase (table 1). Neither 5×10^{-5} M pCMB, nor 7 M urea affect the activity of the immobilized NDP kinase, when acting alone, but when used together there is an activity drop of 85%.

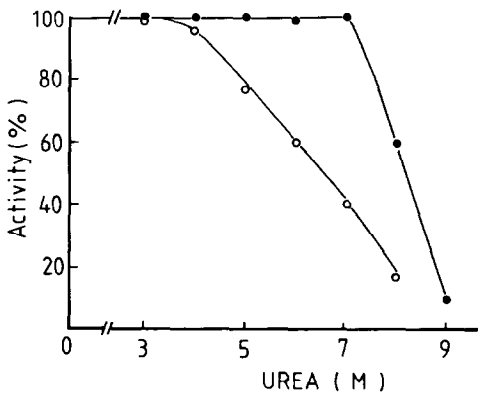

Fig.2. Effect of urea on soluble and Sepharose-bound pig heart NDP kinase. 20 μg of soluble NDP kinase (○) and, respectively, 350 μl of immobilized enzyme corresponding to 35 μl of packed gel (●), were incubated at room temperature for 30 min in 1 ml 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and urea as indicated. 2–10 μl of the incubation mixture were then withdrawn for the measurement of NDP kinase activity. The activity in the absence of urea was considered as 100%.

Table 1
The combined effect of pCMB and urea on pig heart NDP kinase immobilized on Sepharose

pCMB (μM)	Urea (M)	Activity (%)
0	0	100
50	0	100
0	7	100
50	4	100
50	5	75
50	6	60
50	7	15

The experimental conditions are as in the legend to figs.1,2

4. Discussion

The immobilization of the oligomeric enzymes into solid matrices rises special problems as compared to the monomeric enzymes, because their activity generally is dependent on the quaternary structure; the immobilization affects both the conformation of each individual subunit, and their reciprocal interactions to an extent that depends on the number of subunits coupled to the matrix [13,14]. Pig heart NDP kinase is hexameric or octameric as shown by preliminary estimations of molecular weight by gel filtration and polyacrylamide gel electrophoresis. The elution volume of the native enzyme is highly dependent on the composition of the buffer, the presence of bovine serum albumin and substrate nucleotides. This might indicate a lability of the oligomer in dilute protein solutions. The particularly high resistance to denaturation of the immobilized enzyme by urea and temperature indicates that the enzyme is simultaneously coupled via more subunits. However, the enzyme is 'frozen' into a conformation closely resembling that of the native one, since most of the kinetic and molecular properties are preserved.

The differential effect of pCMB on the soluble and matrix-bound NDP kinase may therefore be explained as follows: The mercurial agent blocks an SH group which is buried in the 'normal' subunit conformation or is situated between 2 subunits. Although the enzyme is not dissociated by this treatment [12], the enzymatic activity is impeded. On the other hand, the active conformation of the matrix-bound enzyme is stabilized by the multiple bonds with the matrix. The access of pCMB to the reactive SH group is prevented. In 7 M urea, a partial unfolding of the polypeptide chain

occurs, but the renaturation of the immobilized NDP kinase is very fast, and the enzymatic activity is restored probably in a few seconds [15]. In this denaturating medium (7 M urea), the SH group reacts with pCMB and the enzymatic activity is inhibited.

In conclusion, the immobilization of NDP kinase, although it does not change the kinetic constants, increases the stability towards urea and irreversible inhibitors. The study of the immobilized enzyme (as well as of the soluble cross-linked NDP kinase) may reveal important structural features.

References

[1] Cheng, Y.-C., Robinson, B. and Parks, R. E. (1973) Biochemistry 12, 5–11.
[2] Richard, J.-M. and Colomb, M. (1975) Biochimie 57, 991–993.
[3] Agarwal, R. P. and Parks, R. E. (1971) J. Biol. Chem. 246, 2258–2264.
[4] Cheng, Y.-C., Agarwal, R. P. and Parks, R. E. (1971) Biochemistry 10, 2139–2143.
[5] Mourad, N. and Parks, R. E. (1966) J. Biol. Chem. 241, 271–278.
[6] Lascu, I., Duc, M. and Cristea, A. (1981) Anal. Biochem., in the press.
[7] Lascu, I., Kezdi, M., Goia, I., Jebeleanu, G., Bârzu, O., Pansini, A., Papa, S. and Mantsch, H. H. (1979) Biochemistry 18, 4818–4826.
[8] Ikehara, M., Uesugi, S. and Yoshida, K. (1972) Biochemistry 11, 830–836.
[9] Bârzu, O., Dânşoreanu, M., Munteanu, R., Ana, A. and Bara, A. (1980) Anal. Biochem. 101, 138–147.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Palmieri, R., Yue, R. H., Jacobs, H. K., Maland, L. M., Wu, L. and Kuby, S. A. (1973) J. Biol. Chem. 248, 4486–4499.
[12] Colomb, M. G., Chéruy, A. and Vignais, P. V. (1974) Biochemistry 13, 2269–2277.
[13] Gabel, D. (1973) Eur. J. Biochem. 33, 348–356.
[14] Chan, W. W.-C. (1976) Methods Enzymol. 44, 491–503.
[15] Mozhaev, V. V., Martinek, K. and Berezin, I. V. (1979) Mol. Biol. (Moscow) 13, 73–80.