NDP KINASE: DEMONSTRATION OF PHOSPHORYLATED ENZYME AS THE REACTIVE INTERMEDIATE

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Nucleoside diphosphokinase (NDP kinase), which was first discovered in 1953 (1, 2), and which recently has been obtained in crystalline form from yeast (3), catalyzes the following type of reaction:

$$N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$$

N = a purine or pyrimidine base.

A survey of animal tissues in this laboratory revealed that NDP kinase is in high concentration in human erythrocytes. The enzyme has been isolated from this tissue source in good yield and in a state of purity (65 μmolar units/mg of protein) approximately 1400 fold greater than that of hemolysates.

The reaction catalyzed by erythrocytic NDP kinase has been subjected to a detailed kinetic analysis (4, 5), the results of which indicate that the reaction follows a "ping pong" mechanism (6); i.e. the first substrate adds to and the first product leaves the enzyme surface before the second substrate binds. Figure 1 shows a typical experiment in which the initial reaction velocity was determined with dGDP as the variable substrate and ATP as the changing-fixed substrate. The family of parallel lines seen in this reciprocal plot is characteristic of the "ping pong" mechanism (6).

The observations of Figure 1 and others are consistent with the hypothesis that the erythrocytic NDP kinase reaction occurs with a phosphorylated enzyme as the reactive intermediate. Experiments have now been completed which validate this hypothesis. Purified NDP kinase was reacted with Mg^{++} and ATP-β,γ-P^{32} for one minute, after which time the reaction mixture was subjected to separation on Sephadex G-100 column. There emerged from the column a peak of

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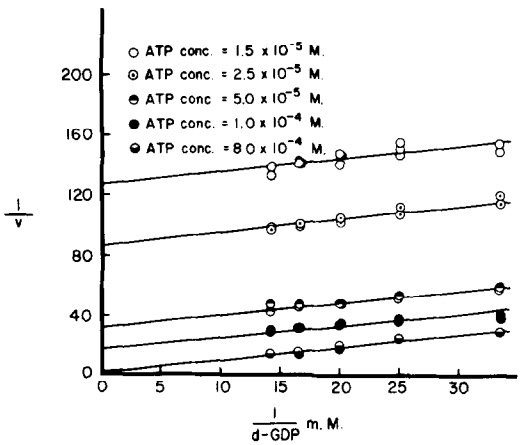

Fig. 1. Plot of reciprocal of the initial velocity ($v = -\Delta OD_{340}/min$) versus reciprocal of concentration of dGDP (millimolar). The reaction components which were held constant are: pyruvic kinase, 0.001 mg (270 μmolar units/mg); lactic dehydrogenase, 0.01 mg (450 μmolar units/mg); phosphoenolpyruvic acid (PEP), 3 mM; DPNH, 0.3 mM; MgCl, 25 mM; KCl, 10 mM; Tris (acetate), 0.15 M, pH. 7.5; NDP kinase, 0.008 μmolar units. dGDP and ATP were added in the concentrations indicated. Volume,1 ml, 30°C.

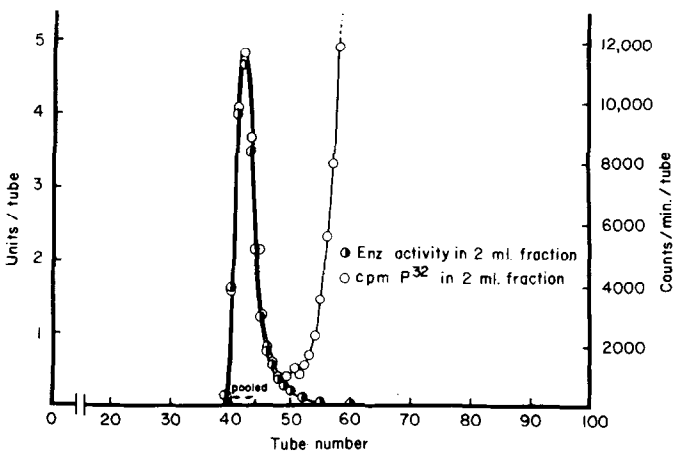

Fig. 2. Separation of Pi^{32}-labeled NDP kinase from ATP^{32} on Sephadex G-25. The reaction mixture in 0.6 ml. consisted of: ATP-γ, β,γ-P^{32} (sp. act. 64 mc/mmole) (Schwartz), 0.75 μmoles; Mg^{++}, 10 μmoles; NDP kinase, 0.5 mg (sp. act. 50 μmolar units/mg); Tris acetate, pH 7.5, 50 μmoles. The reaction mixture was incubated at 30°C for 1 minute. Immediately after the addition of 0.2 ml of EDTA, 0.1 M, and 0.2 ml of 1% serum albumin, the reaction mixture was transfered to a Sephadex G-25 (42 x 2.5 cm) and eluted with 0.1 M Tris acetate pH 7.5 at 80 ml/hour.

radioactivity which was coincident with the peak of enzymic activity. The ratio of P^{32}: NDP kinase activity was remarkably constant throughout the peak. In each of four experiments of this type essentially similar results were obtained, and from $1.1 - 1.6 \times 10^{-4}$ μmoles of P^{32} were fixed per unit of enzyme. To eliminate the possibility that ADP or ATP had bound to the enzyme, the above experiment was repeated with a mixture of ATP-$8C^{14}$ and ATP-β,γ-P^{32}. Only P^{32} emerged from the Sephadex column with the enzyme peak. Preliminary evidence has shown that the P^{32}-labeled protein can be precipitated with 50% acetone or 5% TCA. From the behavior on Sephadex columns the molecular weight of the enzyme is estimated to be somewhat greater than 100,000 gm/mole.

The results of a "bleaching" experiment are presented in Figures 2, 3, and 4. NDP kinase was first isolated in the phosphorylated form (Figure 2). The labeled enzyme was then incubated briefly with GDP and the reaction mixture was again separated on a Sephadex G-25 column (Figure 3). In this case only a small amount of P^{32} remained with the enzyme, and the bulk of the radioactivity emerged in a second peak. An aliquot of the second peak of Figure 3, with added non-labeled orthophosphate, GDP, and GTP was subjected to gradient elution on a DEAE-cellulose (bicarbonate) column (Figure 4). A small amount of P^{32} was found in the orthophosphate peak, but most of the P^{32} emerged in a peak which coincided closely with the GTP peak. The identity of this radioactive substance as GTP was confirmed by chromatography in two solvent systems. Therefore it was concluded that a phosphate group had been transfered from ATP to the enzyme, and, in turn, from the phosphorylated enzyme to GDP to form GTP.

From the above findings it is now possible to delineate the reaction sequence for the erythrocytic NDP kinase reaction:

1. $N_1TP + \text{Enzyme} \rightleftharpoons \text{Enzyme-Pi} + N_1DP$
2. $N_2DP + \text{Enzyme-Pi} \rightleftharpoons N_2TP + \text{Enzyme}$

Whether other enzymes of this class follow a similar mechanism remains to be determined. Also of interest is the possible identity of NDP kinase with certain of the "coupling factors" reported to play a role in oxidative phosphorylation. An important question under study

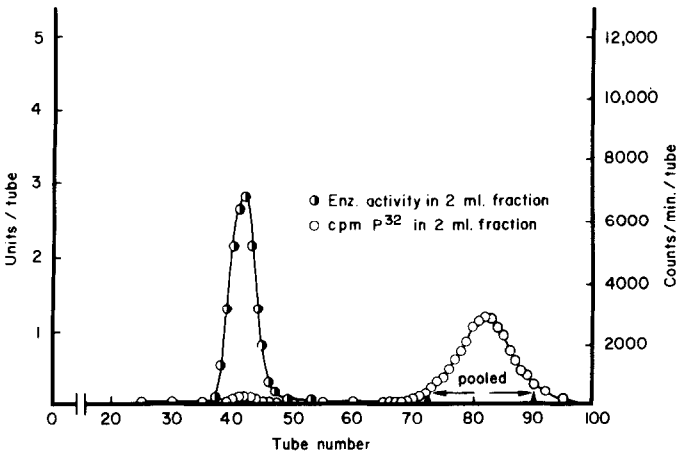

Fig. 3. Separation of NDP kinase from GTP^{32} on Sephadex G-25. The phosphorylated NDP kinase in tubes 40 through 44 (Fig. 2), (about 15 μmolar units of NDP kinase activity in 10 ml) was incubated for 1 minute at 30°C with 1.5 μmoles of GDP and 10 μmoles of Mg^{++}. After addition of EDTA, 20 μmoles, and 0.2 ml of 1% serum albumin, the reaction mixture was added to a Sephadex G-25 column (42 x 2.5 cm) and eluted with 0.1 M Tris acetate, pH 7.5 at 80 ml/hour. Two ml fractions were collected.

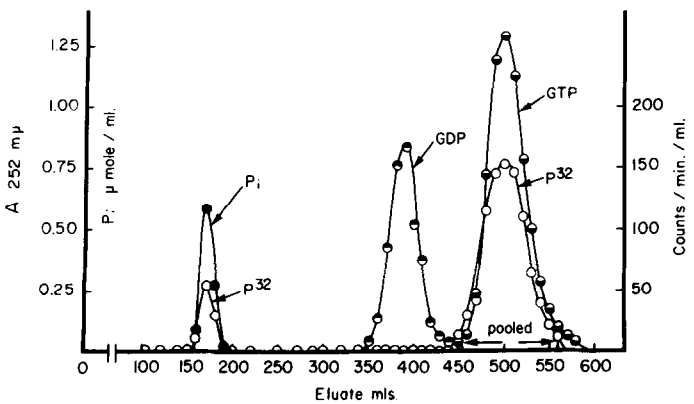

Fig. 4. Separation of Pi, GDP, and GTP on DEAE-cellulose (bicarbonate). Non-labeled orthophosphate, 10 μmoles; GDP, 2 μmoles; and GTP, 6 μmoles, were added to an aliquot of the pooled fractions, 71 through 90 (Fig. 3). The resulting mixture was added to a DEAE-cellulose (bicarbonate) column (20 x 2.5 cm) and elution was performed by a linear gradient (0.2 M - 0.4 M triethylammonium bicarbonate, vol 800 ml) (7).

The third peak was pooled, evaporated to dryness and subjected to paper chromatography in two solvent systems, isobutyric acid:NH_4OH: water (66:1:33) and isopropanol: 1% ammonium sulfate:0.1M EDTA (200:100:3).

is the nature of the Pi to enzyme linkage; e.g. is phosphohistidine (8) involved?

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BIBLIOGRAPHY

1. Krebs, H.A., and Hems, R. BBA, 12, 172 (1953).
2. Berg, P. and Joklik, W.K., Nature, 172, 1008 (1953).
3. Ratliff, Robert L., Weaver, Robert H., Lardy, Henry A., and Kuby, A. Stephen, JBC, 239, 30 (1964).
4. Mourad, Nabeeh, and Parks, R.E., Jr, Proc. Am. Assoc. Cancer Res., Vol. 6 (1965).
5. Cha, Sungman, Mourad, Nabeeh, Cha, C-J. M., Parks, R.E., Jr. Fed. Proc. (Abstract) in press (1965).
6. Cleland, W.W., Biochim. et Biophys. Acta, 67, 104, 173, 188, (1963).
7. Cha, Sungman, and Parks, R.E., Jr. JBC, 239, 1961 (1964).
8. Boyer, P.D., Science, 141, 1147 (1963).