EVIDENCE FOR AN INTERMEDIARY PHOSPHORYLATION OF NUCLEOSIDE DIPHOSPHATE KINASE FROM PEA SEED

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1. Introduction

Several nucloside diphosphate kinases (EC 2.7.4.6) have been shown to be phosphorylated by $AT^{32}P$, among them the enzyme from pea seed [1]. The degradation products obtained after alkaline hydrolysis of ^{32}P-labelled NDP kinases suggest that the phosphoryl group is bound to the N-1 position of an imidazol group in the enzyme [1–3].

Kinetic data obtained with NDP kinases from different sources indicate that during catalysis the enzyme exists in two inter-convertable and stable forms, since the mechanism is of the ping-pong type [4–6].

A prerequisite for such a mechanism is that the phosphorylation as well as the dephosphorylation reactions are at least as rapid as the overall reaction. Evidence for this has been presented for the bovine liver enzyme by using a rapid mixing technique [7]. It was, however, of interest to investigate whether this mechanism was valid also for NDP kinases from other sources. In the present work the enzyme from pea seed has been subjected to such an investigation. It has been possible to design the experiments so that all reaction rates studied were determined using the rapid mixing device. The pea seed enzyme was chosen since it represents a phylogenetically different source and can be obtained in reasonable quantities.

In the case of the bovine liver enzyme the rate of the partial reactions was determined in a rapid mixing apparatus, while the rat of the overall reaction was measured spectrophotometrically by a coupled pyruvate kinase–lactate dehydrogenase method [7]. The rapid mixing experiments were performed in the presence of the components of the coupled assay in order to obtain as similar conditions in the two types of experiment as possible.

The results with the bovine liver enzyme suggested, however, that the components of the coupled assay were inhibitory to the enzyme at least under the conditions used. When starting the present investigation this inhibition was also found with the pea seed enzyme. Therefore the overall reaction was measured in the rapid mixing apparatus using no other components than those of importance for the NDP kinase reaction itself.

All data including initial velocity analysis of substrate inhibition are in accordance with the view that the phosphorylated enzyme of pea seed NDP kinase is an intermediate in enzyme catalysis

2. Materials and methods

Sephadex G-50 was obtained from Pharmacia, Sweden. The nucleotides and phosphoenolpyruvate were from Sigma. Pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) were purchased from Boehringer. All reagents were of analytical grade.

NDP kinase was prepared from dried peas as dedescribed previously [1]. The specific activity of the enzyme preparation used in this study was 1200 units per mg [γ-^{32}]ATP was prepared as described by Engström [8]. The [^{32}P]orthophosphate content of the $AT^{32}P$ was 5%. In the rapid mixing experiments the $AT^{32}P$ was diluted with unlabelled ATP giving a specific activity of 30 000–100 000 cpm/nmole.

Not only the phosphorylation and dephosphorylation reactions but also the overall reaction was studied in the rapid mixing apparatus. This was constructed essentially as described previously [7]. The syringes were, however, not emptied by a steel spring but by hydraulic pressure and the volume ejected from each syringe was

1.0 ml [9]. The enzyme concentration in the mixing chamber was 0.015 mg/ml. At this concentration the enzyme activity was not affected by the mixing procedure. Thus, there was no need for an addition of serum albumin as in the case of the bovine liver enzyme.

The phosphorylation reaction was carried out in the apparatus essentially as described previously [7]. One syringe contained NDP-kinase 0.03 mg/ml in 0.05 M Tris–acetic acid buffer, pH 7.5. The other syringe contained 30 μM AT^{32}P and 200 μM $MgCl_2$ in the same buffer. After mixing in the apparatus the solution was quenched in 5 ml 0.15 M NaOH. SDS was added, the samples were neutralized and the ^{32}P-labelled enzyme was then islated by chromatography on Sephadex G-50 as described before [7]. The lability in acid of the ^{32}P-phosphoryl bond was determined as before [10].

The effect on the phosphorylation reaction of the addition of the components of the coupled NDP-kinase assay was also studied. The components were added to the solution of each syringe in the following concentrations: 3 mM PEP, 0.2 mM NADH, 25 mM $MgCl_2$, 10 mM KCl, 0.01 mg/ml pyruvate kinase and 0.03 mg/ml lactate dehydrogenase.

For the dephosphorylation experiments the ^{32}P-labelled enzyme was prepared as described previously [7]. It was used immediately after the ^{32}P-labelling. One syringe was filled with a solution of the ^{32}P-labelled enzyme (0.03 mg/ml) in 0.05 M Tris–acetic acid buffer, pH 7.5. The other syringe contained 24 μM GDP and 200 μM $MgCl_2$. After mixing the quenching was performed as above, as well as the isolation of the remaining ^{32}P-labelled enzyme.

The rate of the overall reaction was also studied in the rapid mixing apparatus, i.e. under the same conditions as the partial reactions. One syringe was filled with NDP-kinase 0.03 mg/ml, the other with 30 μM AT^{32}P, 24 μM GDP and 200 μM $MgCl_2$. All reactants were in 0.05 M Tris–acetic acid buffer, pH 7.5. GDP was used as nucleoside diphosphate and not dGDP as in the report on the bovine liver enzyme, since deoxynucleotides are not stable during the following Dowex chromatography. After mixing in the apparatus the reaction was quenched in 5 ml of 0.1 M NaOH. One min later 1.2 ml of 0.5 M HCOOH was added. One μmole of unlabelled ATP and GDP were added to each sample which were then applied to columns (1.3 cm × 3.8 cm) of Dowex 1 × 8 in formate form. The ^{32}P-orthophosphate was washed out with 4 M HCOOH. AT^{32}P was eluted with 0.25 M ammonium formate in 4 M HCOOH and GT^{32}P with 0.8 M ammonium formate in 4 M HCOOH. The total amount of radioactive nucleotides applied to the columns were calculated from the specific radioactivities of each nucleotide fraction and the amount of carrier nucleotide added. The molar extinction coefficient used for ATP and GTP were 14.3×10^3 and 11.8×10^3, respectively [11].

The number of active sites per enzyme molecule in the enzyme preparation used was determined from the molar incorporation of [^{32}P]orthophosphate in the enzyme [1]. The incorporation of [^{32}P]orthophosphate was about 2 moles per 70 000 *g* at concentrations of AT^{32}P from 15 μM to 1 mM. This was somewhat less than the value (2.6 to 3.1) obtained with the best preparations [1].

The kinetic behaviour of the enzyme was investigated by initial velocity analysis at different concentrations of ATP and dGDP. This was performed by the coupled pyruvate kinase–lactate dehydrogenase method of Mourad and Parks [4].

3. Results and discussion

In the report on the bovine liver enzyme the components of the coupled NDP-kinase assay were found to be slightly inhibitory to the phosphorylation reaction. In that case the components were added only to the syringe containing AT^{32}P. When, however, in a similar experiment on the pea seed enzyme these components were also added to the syringe containing NDP-kinase, the phosphorylation reaction was too slow to be measured in the apparatus. NADH seemed to be one of the inhibitory components since when it was omitted the phosphorylation rate increased considerably. To avoid these inhibitory effects all reaction rates for the pea seed enzyme were studied in the presence of the NDP-kinase substrates only.

The rate of the phosphorylation reaction is shown as a first order plot in fig. 1A. The pseudo first order rate constant was 90 sec^{-1}, which would allow a turnover number per active site of enzyme of 5400 min^{-1}. The acid-lability of the phosphorylated enzyme was the same whether the incubation time was a few msec or several seconds. This indicates that the same kind

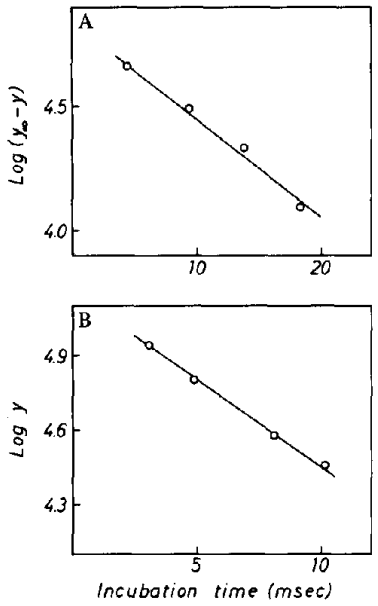

Fig. 1. First order plots of the phosphorylation (A) and dephosphorylation reaction (B) of pea seed NDP-kinase. Y represents the [^{32}P]phosphate bound to the enzyme after different incubation times, y_∞ the maximal amount of [^{32}P]phosphate bound to the enzyme [7]. Each point is the mean of two separate determinations. For details see under Materials and methods.

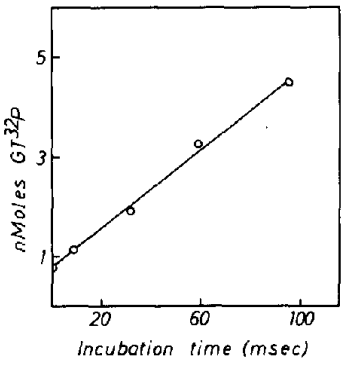

Fig. 2. Formation of GT^{32}P during short-time incubation of NDP-kinase with AT^{32}P and GDP in the rapid mixing apparatus. The amount of GT^{32}P refer to 2 ml of incubation mixture. For details see under Materials and methods.

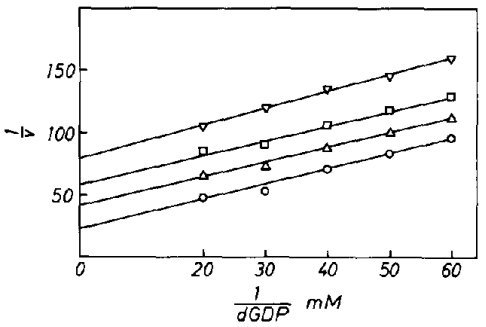

Fig. 3. Plot of the reciprocal of the initial velocity ($-\Delta E_{340}$/min) of the NDP-kinase reaction against the reciprocal of the dGDP-concentration at various concentrations of ATP. The coupled pyruvate kinase–lactate dehydrogenase method of Mourad and Parks [4] was used. The concentration of NDP-kinase was 0.013 μg/ml, that of pyruvate kinase and lactate dehydrogenase 2 μg/ml and 6 μg/ml, respectively. The lines were drawn by the least squares method. The ATP concentrations were varied as follows: (▽—▽—▽) 0.040 mM; (□—□—□) 0.075 mM; (△—△—△) 0.1 mM; (○—○—○) 2 mM.

of phosphoryl enzyme was formed irrespectively of the incubation time.

The dephosphorylation reaction is shown as a first order plot in fig. 1B. A pseudo first order rate constant of 170 sec^{-1} was obtained from this plot. This would permit a turnover number of 10 000 per active site of enzyme. In the previous report on the bovine liver enzyme only a very approximate value could be obtained for the dephosphorylation rate [7].

The overall NDP-kinase reaction was measured by determining the amounts of GT^{32}P formed from AT^{32}P and GDP after short duration incubation in the rapid mixing device. The amount of GT^{32}P formed was linear with time until about 20% of the AT^{32}P was consumed (fig. 2). The turnover number per active site for the overall reaction was found to be 2700 min^{-1}.

The turnover numbers obtained for the partial reactions can be directly compared to that of the overall reaction since all reactions were studied in the rapid mixing apparatus under almost identical conditions. It can thus be concluded that the phosphorylation and dephosphorylation reactions are at least as rapid as the overall reaction. This is in accordance with the view that the phosphoryl enzyme is a true intermediate in the enzyme reaction. The reaction rates are about the same as those obtained for the bovine liver enzyme.

The result of the initial velocity analysis was also in accordance with the view that the phosphoryl enzyme is a true intermediate. In fig. 3 the reciprocal of the velocity of the NDP-kinase reaction at different

ATP concentrations has been plotted against the reciprocal of the dGDP concentration. A family of parallel lines was obtained indicating that the enzyme kinetics is of the ping-pong type. All NDP kinases so far tested have been shown to be phosphorylated by their nucleoside triphosphate substrate. For references see [1, 2, 4–7].

It is shown in this report and the previous one [7] that the phosphorylated enzyme of two quite different NDP-kinases both have the characteristics of an enzyme intermediate. It is thus reasonable to suppose that the phosphoryl enzymes obtained from other NDP-kinases also represent true enzyme intermediates.

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