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Nucleoside Diphosphokinase from Beef Heart Cytosol. II. Characterization of the Phosphorylated Intermediate†

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ABSTRACT: Beef heart cytosol NDP-kinase was phosphorylated by [^{32}P]ATP and the bound [^{32}P]phosphate could be discharged on ADP. The phosphorylation and dephosphorylation steps depended on Mg^{2+}, as assessed in rapid-flow experiments. These findings are in good agreement with kinetic results presented in the preceding paper. Phospho-NDP-kinase exhibited the same behavior as the free enzyme on sucrose gradient sedimentation and gel filtration, however, it could be differentiated by its more acid isoelectric point (5.9 instead of 8.5). Coelectrophoresis and cochromatography of alkaline hydrolysates of phospho-NDP-kinase, with reference to standard phosphoamino acids, allowed the identification of 3-phosphohistidine and traces of 1-phosphohistidine in the degradation products. A study of the acid lability of the phosphate linkage in the phosphoenzyme indicated that in native phospho-NDP-kinase, phosphate was bound to the N_1 of the imidazole ring of histidine and that 1-phosphohistidine could be isomerized into 3-phosphohistidine in an alkaline medium. Consistent with the phosphorylation of histidine and its presumed localization at the active site of the enzyme, was the finding that diethyl pyrocarbonate inhibited the exchange reaction and that inhibition was released by hydroxylamine. The enzyme was also inactivated by photooxidation in the presence of Rose Bengal; protection against photooxidation was afforded by ADP or ATP but not by 5′-adenylylmethylenediphosphonate, a methylene analog of ATP which is not a substrate.

In the preceding paper (Colomb *et al.*, 1972), we describe the purification of a nucleoside diphophokinase[1] isolated from beef heart cytosol, its molecular characteristics, and kinetic properties. Here we report physical and chemical studies on the phosphorylated intermediate of this enzyme.

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[1] Abbreviations used are: NDP-kinase and phospho-NDP-kinase, nucleoside diphophokinase and its phosphorylated derivative; AOPCP, adenosine 5′-methylenediphosphonate; AOPOPCP, 5′-adenylylmethylenediphosphonate.

Methods

Preparation of the N-Phosphorylated Derivatives of [^{14}C]-Lysine, [^{14}C]Hydroxylysine, and [^{14}C]Arginine. N^3-Phospho[^{14}C]histidine, N^{ϵ}-phospho[^{14}C]lysine, N^{ϵ}-phospho[^{14}C]-hydroxylysine, and N^{ϵ}-phospho[^{14}C]arginine were synthesized from the corresponding [^{14}C]amino acids and phosphorus oxychloride according to Hultquist (1968) and Zetterqvist and Engström (1967). The [^{14}C]amino acids used in this study were obtained from the Commissariat à l'Energie Atomique (Saclay).

The original procedures were followed up to the purification step. The crude phospho amino acids were then purified by column chromatography on DEAE-cellulose instead of Dowex 1. Starting for instance from 50 mg of crude phospho amino acid, we used about 6 g of DEAE-cellulose (0.88 mequiv/g). The DEAE-cellulose was previously equilibrated in a column with 20 mM Tris-HCl (pH 8), the aqueous solution of crude phospho amino acid was brought to pH 8 and its ionic strength was adjusted to match the equilibrium conditions of the DEAE-cellulose. The elution was carried out with a continuous gradient ranging from 20 mM Tris-HCl (pH 8) to 100 mM NaCl–20 mM Tris-HCl (pH 8) followed by a final addition of 100 mM NaCl–20 mM Tris-HCl (pH 8). The phospho [^{14}C]amino acids were detected in small aliquots of eluate spotted on Whatman No. 1 paper by two separate tests using ninhydrin and molybdic acid, respectively. Other aliquots were used for ^{14}C scintillation counting. A purity control was performed by high-voltage electrophoresis in a borate buffer (pH 8.5) followed by ninhydrin detection and autoradiography. The order of migration from the slowest compound was: phospholysine, phosphoarginine, phosphohydroxylysine, and 3-phosphohistidine (*cf.* Figure 6B). Except for N^{ϵ}-phospho[^{14}C]hydroxylysine which appears to be a mixture of two isomers, all other phospho[^{14}C]amino acids were homogeneous. Their specific radioactivity ranged from 230 to 256 dpm per nmole except that of histidyl phosphate which was half as much.

Starting from phosphorus oxychloride and histidine Hultquist (1968) isolated phosphorylated derivatives among which were the 1,3-diphosphohistidine and the 1- and 3-phosphohistidine. The phosphohistidine species that we obtained after purification was identified by measuring the amount of phosphate fixed on the [^{14}C]histidine after isolation of the phospho derivative by electrophoresis and elution of the area revealed by autoradiography. The microassay of Itaya and Ui (1966) which allowed the estimation of 0.1 μg of phosphorus was used for phosphate determination. A phosphate to histidine molar ratio of 1 was found. Finally from the stability characteristics reported for the different phosphohistidine species (Hultquist *et al.*, 1966) it was concluded that the compound we had purified was 3-phosphohistidine. Minute amounts of a compound with a mobility similar to that of 1-phosphohisitine (Zetterqvist, 1967) could be detected in crude preparations of 3-phosphohistidine and located on the electrophoretogram at pH 8.5 at the same level as phosphohydroxylysine between 3-phosphohistidine and phospholysine (*cf.* Figure 6B).

Hydrolysis of Phospho-NDP-kinase. Labeling of NDP-kinase by [^{32}P]phosphate was achieved by incubation of the enzyme with [γ-^{32}P]ATP and $MgCl_2$ as detailed in Results. The radioactive protein was separated from ADP and the nonreacted [γ-^{32}P]ATP by chromatography on Bio-Gel P4 previously equilibrated with 0.1 M phosphate buffer (pH 7); it was hydrolyzed by 3 M KOH in sealed tubes at 100° for 3 hr. After cooling, the hydrolysate was desalted by contact with Dowex 50 (H^+ form) added stepwise until a pH of 7 was reached. After a 10-min centrifugation at 10,000*g*, the supernatant was recovered for identification of the [^{32}P]phospho amino acids.

Preparation of Antibodies. The technique of Neuhaus as described by Demaille (1965) was used. After initial intravenous injections of Zymosan for 3 consecutive days, rabbits received, 10 days later and for 4 consecutive days, intramuscular injections of 20 mg of NDP-kinase followed 1 week later by a final injection of 100 mg of enzyme. Freund's complete adjuvant was incorporated to the injected NDP-kinase preparations. Twenty days after the last injection the rabbits were bled. The sera were used in immunochemical identification experiments using the Ouchterlony (1949) double-diffusion technique.

Photooxidation. The conditions described by Westhead for enolase (1965) were used for photooxidizing NDP-kinase. Irradiations were performed by means of two 250-W tungsten lamps located at 18 cm on each side of a cylindrical reaction vessel (volume 1.5 ml) thermostated at 25°. The reaction was carried out under magnetic stirring in the presence of 10^{-5} M Rose Bengal and in some cases of 10^{-4} M EDTA, with constant air bubbling in the reaction medium. The validity of this device was checked by recording with a GME oxygraph the oxygen consumption resulting from the photooxidation of solutions of histidine and of methionine in the absence of air bubbling; in this case only the dissolved oxygen was consumed. To evaluate the loss of activity strictly due to photooxidation, a parallel incubation was run in the dark to account for the loss of activity upon dilution and standing. Freshly prepared NDP-kinase was used for these experiments. Solutions of NDP-kinase stored in the presence of azide were avoided since azide is liable to absorb light energy.

Rapid-Flow Measurements. Several experiments on the phosphorylation and the dephosphorylation steps of the transphosphorylation reaction were performed with a homemade rapid-flow apparatus. The basic elements of this device are essentially the same as those described by Sturtevant (1964) for a stopped-flow system. The plungers of two driving syringes were pushed upward through the action of a steel spring. The two syringes were forced to deliver fixed volumes from 0.6 to 3 ml through four jets into a mixing chamber. The reaction took place in the mixing chamber and went on in the outlet tubing connected to the chamber until the mixture reached the quenching solution. The volume of the quenching solution was routinely 2 ml, the volume injected in it varied from 0.4 to 0.6 ml for reaction times between 8 and 20 msec. To allow a better mixing and a good reproducibility the quenching solution was placed in a plastic syringe whose piston was able to absorb by displacement without appreciable resistance the transient increase of pressure.

An original optical device was used for monitoring the displacement of the plungers moved by the spring. An opaque screen rigidly linked to the pushing carriage of the driving syringes was placed between the linear and vertical filament of a tungsten lamp and a photocell. The movement of the screen intercepted the light beam emitted by the lamp, resulting in an immediate and linear change of the electrical signal collected by the photocell. A direct-recording oscillograph (Honeywell visicorder) connected to the photocell allowed a recording of the movement of the screen as a function of time and an exact determination of the velocity of the plungers. For a given spring this velocity was found constant. From this velocity and the diameter and the length of the tubing it was

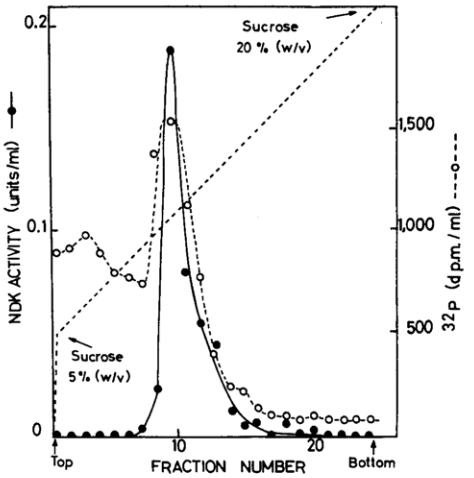


FIGURE 1: Sucrose gradient centrifugation pattern of [^{32}P]phospho-NDP-kinase. Centrifugation was carried out as described in Methods; 0.2-ml fractions were collected for assay of NDP-kinase activity and determination of radioactivity.

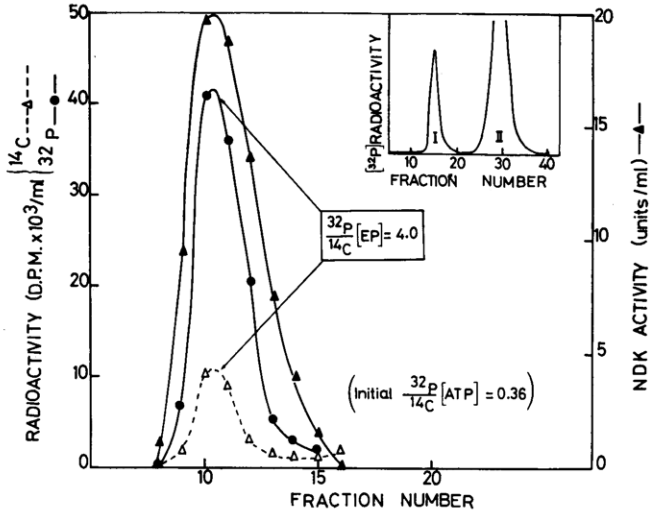


FIGURE 3: Evidence for phosphorylation of NDP-kinase by use of [^{14}C,γ-^{32}P]ATP. NDP-kinase (0.97 mg) was incubated with 1.3 μmoles of $MgCl_2$, 0.8 μmole of [^{32}P,^{14}C]ATP, 38,000 dpm/nmole of ^{32}P, and 106,000 dpm/nmole of ^{14}C in 1 ml of 0.1 M phosphate buffer (pH 7) for 5 min at 20°. The separation of the labeled enzyme was carried out on Bio-Gel P4, equilibrated with 0.1 M phosphate buffer (pH 7). The ^{32}P and ^{14}C radioactivities were determined by scintillation counting. The elution profile (*insert*) points to the satisfactory separation on Bio-Gel P4 of [^{32}P]phospho-NDP-kinase (peak I) from [^{32}P]ATP (peak II).

easy to calculate reaction times. Our apparatus was calibrated for four different springs and for outlet tubing lengths varying from 4 to 30 cm; its working range was usually between 8 and 60 msec.

Other Methods. Zone sedimentation in sucrose gradient, gel filtration, electrofocusing, and the assay of NDP-kinase activity have been described in the preceding paper (Colomb *et al.*, 1972).

Results

Phosphorylation of NDP-kinase with [^{32}P]ATP. The Ping-Pong kinetics displayed by beef heart cytosol NDP-kinase (see Colomb *et al.*, 1972) is readily explained by a stable phosphorylated form of NDP-kinase. The experiments to be described now provide a direct demonstration of a phosphorylated form of this enzyme. About 1 mg of purified NDP-kinase was incubated in 1 ml of 0.1 M phosphate (pH 7) with 0.3 mM [γ-^{32}P]ATP and 0.6 mM $MgCl_2$ for 15 sec at 28°.

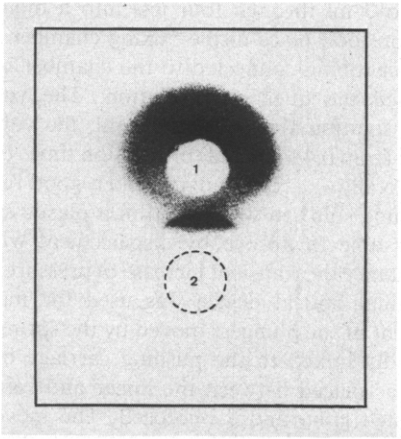


FIGURE 2: Autoradiogram of an immunodiffusion plate where [^{32}P]phospho-NDP-kinase was reacted against an NDP-kinase antibody. A 0.9% agarose gel in 0.1 M phosphate buffer (pH 8) and 0.04% azide was used for the diffusion. The [^{32}P]phospho-NDP-kinase preparation and the antiserum (see Methods) were placed in two circular wells of 6-mm diameter (numbered 1 and 2, respectively) and left to diffuse for 24 hr at 20°. After rapid washing (6 hr) with a 0.9% NaCl solution, they were placed in contact with X-ray film for 48 hr.

After cooling, the incubation medium was applied to a column of Bio-Gel P4 (1.4 × 40 cm) equilibrated with 0.1 M phosphate buffer (pH 7) at 2°. Elution was carried out with the same buffer. A ^{32}P-labeled protein containing the whole NDP-kinase activity was eluted as a single peak quite distinct from the [^{32}P]ATP peak (see insert in Figure 3). An aliquot of this fraction was centrifuged through a sucrose density gradient. Here again, one peak of enzyme activity was obtained, coincident with the peak of radioactivity (Figure 1). The radioactivity remaining at the top of the gradient is probably due to degradation products.

An additional identification test was performed, based on immunological properties of the phosphorylated NDP-kinase. Antibodies made to purified NDP-kinase were tested using [^{32}P]phospho-NDP-kinase as antigen. On double diffusion a single immunoprecipitate was formed, coincident with a ^{32}P precipitin band as revealed by autoradiography (Figure 2).

That the ^{32}P labeling of NDP-kinase involved a phosphate transfer from [^{32}P]ATP to the enzyme and was not due to the binding of the whole ATP molecule was clearly shown by incubating the enzyme with a doubly labeled [^{14}C,γ-^{32}P]ATP. In the experiment illustrated in Figure 3, the ratio of ^{32}P to ^{14}C radioactivities in ATP was equal to 0.36. After incubation, the labeled enzyme was separated from unreacted ATP by chromatography on Bio-Gel P4 and tested for ^{14}C and ^{32}P activities. The radioactivity ratio ^{32}P/^{14}C of the NDP-kinase peak was 11 times as high as in the initial [^{14}C,^{32}P]-ATP, indicating that more than 90% of the enzyme-bound radioactivity was accounted for by bound [^{32}P]phosphate.

In routine phosphorylation experiments, beef heart cytosol NDP-kinase was found to bind between 0.4 and 1.2 moles of phosphate per 100,000g of protein. However, optimum conditions for NDP-kinase phosphorylation have not been thoroughly investigated and it is probable that the maximal amount of bound phosphate is higher. The bound [^{32}P]phos-

phate could be entirely discharged by ADP, UDP, GDP, CDP, dADP, and AOPCP, but not at all by cAMP and glucose, pointing to the specificity of the phosphate transfer and to the function of the phospho-NDP-kinase as an enzyme intermediate in the transphosphorylation reaction.

Physical Properties of Phospho-NDP-kinase. The free and phosphorylated enzymes were apparently similar with respect to size and shape but electrofocusing revealed differences in their electrophoretic behavior. On electrofocusing in a pH 3–10 ampholines gradient the [^{32}P]phosphorylated NDP-kinase exhibited two peaks of enzyme activity at pH 8.3 and 6.2 and only one peak of radioactivity at pH 6.2 (Figure 4A). Above data had shown that the enzyme-bound radioactivity was mostly due to bound [^{32}P]phosphate. Assuming that the pH 6.2 peak corresponds mainly to bound [^{32}P]phosphate and the pH 8.3 peak to unphosphorylated NDP-kinase (*cf.* physical data on "free" enzyme in Colomb *et al.*, 1972), it is concluded that the isoelectric point of the phosphoenzyme is markedly more acid than that of the free one. In another experiment the electrophoresis column was perfused with ATP and $MgCl_2$ to maintain during electrofocusing a steady concentration of phosphorylated NDP-kinase (Figure 4B). In this case, a single sharp peak of enzyme activity at pH 5.9 was found in agreement with the accumulation of the phosphoenzyme under these conditions. The large shift (more than 2 pH units) of the isoelectric point toward lower pH values which occurs during the phosphorylation of the enzyme is hardly accounted for by phosphate binding alone (of the order of 1 mole/mole of NDP-kinase in our experimental conditions) and is probably due to subsequent conformational changes altering the apparent charge of the molecule.

Rapid-Flow Experiments on the Phosphorylation and Dephosphorylation Reactions. The apparatus described under experimental procedures was used for investigations on both steps of the transphosphorylation reaction. As shown in Figure 5A, the phosphorylation of the cytosolic NDP-kinase by ATP is a Mg^{2+}-dependent process; virtually no phosphoenzyme accumulated when $MgCl_2$ was replaced by EDTA. At the pH of the incubation medium in this experiment (pH 7) 58 and 66% ATP were Mg bound when the $MgCl_2$ to ATP ratios were 2 and 6, respectively; increasing the $MgCl_2$ to ATP ratio from 2 to 6 increased by 20% the amount of enzyme-bound phosphate, in good agreement with the expected function of Mg-ATP as phosphate donor. By incubation with 0.05 mM [^{32}P]ATP in the presence of $MgCl_2$, 80% maximal level of phosphorylation was reached in 10 msec when the reaction was stopped by alkali (Figure 5B). All other conditions remaining the same, 50% only of the maximal phosphorylation was obtained in 10 msec when acidification instead of alkalinization was used to stop the enzyme phosphorylation (Figure 5D). The two latter results clearly point to the acid-labile, alkali-stable character of the phosphate bond in phospho-NDP-kinase. The effect of ATP concentration on the rate of NDP-kinase phosphorylation is compared in Figure 5C,D. Decreasing the ATP concentration from 0.050 to 0.015 mM resulted in a decreased rate of phosphorylation, with a half-maximal phosphorylation being reached in 15 msec instead of 10 msec.

The phosphorylated NDP-kinase rapidly discharged its phosphate on ADP to provide ATP; more than 80% protein-bound phosphate was transferred to ADP in less than 10 msec (Table I). A maximal rate of transfer was observed in the absence of added $MgCl_2$. However, it is likely that the enzyme solution used in this experiment contained traces of

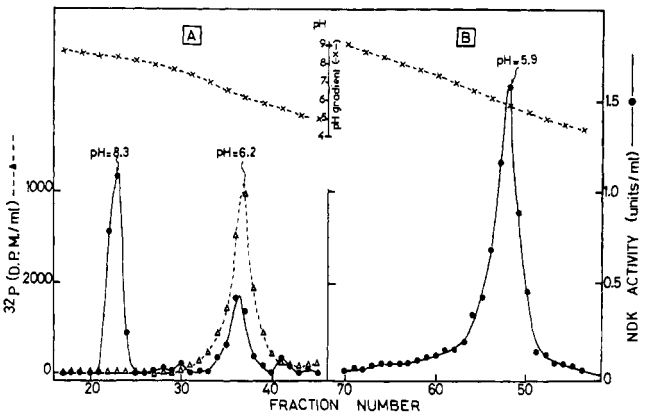


FIGURE 4: Electrofocusing of phospho-NDP-kinase. (A) Phospho-NDP-kinase was introduced to a preformed electrophoresis gradient so as to minimize any extent of decomposition. The procedure carried out was as follows. The pH gradient was formed in a prerun electrophoresis carried out with 1% ampholine, pH 3–10 in a 0–45% discontinuous sucrose gradient. The cathode was at the bottom of the column. After 40-hr focusing the electric power was switched off. A thin polyethylene tube was then introduced into the column to a distance of two-thirds of the gradient length from the anode side. A volume of 1.5 ml of the gradient was pumped out carefully and the density of the fraction was determined by weighing a known aliquot; the pH was 6.8. The density of 1 ml of a solution of [^{32}P]phospho-NDP-kinase (3×10^5 dpm) was brought to the same density as that of the pumped gradient fraction by addition of solid sucrose (the amount of sucrose to be added was calculated from the tables for specific gravity of aqueous sugar solutions in the Handbook of Physics and Chemistry (1963). The entire sample of phospho-NDP-kinase was introduced in the column by the polyethylene tube avoiding any bubble. After removing the tube, the power (800 V) was applied for 6 hr. After elution, the pH and the enzyme activity were measured on the collected fractions (1.5 ml). Aliquots were withdrawn for radioactivity counting. (B) The discontinuous 0–45% sucrose gradient containing 1% ampholine, pH 3–10, included 0.1 mM ATP and 1 mM $MgCl_2$. During the course of gradient preparation, 33 units of NDP-kinase was introduced in 1% ampholine–15% sucrose. The cathode was at the top of the column. The cathode solution was made of 2% ethanolamine, 0.1 mM ATP, and 1 mM $MgCl_2$. During the electrophoresis run (40 hr) a continuous flow of cooled cathode solution (1.6 ml/hr) was maintained at the cathode side. After focusing, 1.5-ml fractions were collected for pH and activity measurements. It was verified by separate chromatographic tests (Duée, 1968) that ATP was not degraded during the electrophoresis run.

Mg^{2+} or other equally efficient metal ions since added EDTA completely blocked the reaction. Interestingly, addition of $MgCl_2$ in large excess with respect to ADP slowed down by 50% the phosphate transfer. Thus, whereas excess Mg^{2+} undoubtedly interferes with the dephosphorylation step of the exchange reaction, free ADP does not stand as phosphate acceptor for the phosphorylated NDP-kinase from beef heart cytosol, in agreement with kinetic data.

Identification of Phosphorylated Amino Acids in Phospho-NDP-kinase Hydrolysates. A preliminary study of the pH effect on the release of inorganic phosphate from phospho-NDP-kinase had revealed that the phosphate links in the phosphoenzyme are more stable at alkaline pH than at acid pH. Likely candidates as acid-labile, alkali-stable phospho amino acids are 1- or 3-phosphohistidine, N^{ϵ}-phospholysine, and *N*-phosphoarginine. N^{ϵ}-Phosphohydroxylysine, although a most improbable candidate in view of the virtually exclusive occurence of hydroxylysine in collagen, was nevertheless included in identification tests. Identification of [^{32}P]phospho amino acids in alkaline hydrolysates of [^{32}P]phospho-NDP-

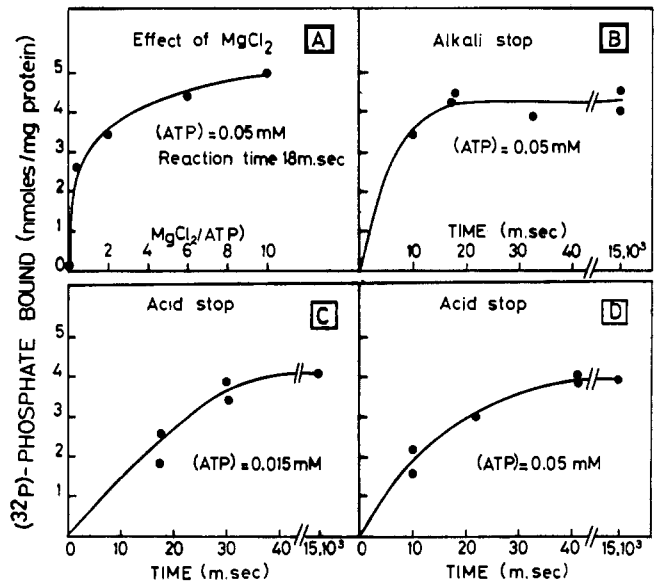


FIGURE 5: Phosphorylation of NDP-kinase with [^{32}P]ATP. Measurements by rapid-flow method. (A) NDP-kinase (1.8 ml, 0.16 mg/ml) in 0.05 M phosphate buffer (pH 7) was mixed with an equal volume of 0.05 mM ATP and various concentrations of $MgCl_2$ in 0.05 M phosphate buffer (pH 7) in the rapid-flow apparatus at 20°. After 18 ± 0.5 msec, 0.6 ml of reaction medium was expelled into 2 ml of an alkaline quenching medium made of 0.7 M KOH and 0.05 M phosphate buffer (pH 7). Three aliquots of 0.1 ml of this mixture were then filtered on 0.22 μ Millipore membranes which retained all NDP-kinase activity. After three washings with 0.25 ml of the quenching medium, the radioactivity remaining on the filters and corresponding to [^{32}P]phospho-NDP-kinase was counted in a gas-flow counter. (B) NDP-kinase was made to react with 0.05 mM ATP and 0.3 mM $MgCl_2$ for different periods of time obtained by varying the length of the outlet tube (*cf.* Methods). The reaction was stopped as in part A. (C and D) In expt C, NDP-kinase reacted with 0.015 mM ATP and 0.09 mM $MgCl_2$ and in expt D with 0.05 mM ATP and 0.3 mM $MgCl_2$. The reaction was stopped as in part A except that the quenching medium was an acid medium made of 0.43 M citrate–0.05 M phosphate buffer (pH 3.3). In separate experiments, periods of incubation of 15 sec were used to check the plateau phase obtained in rapid flow at times longer than 30–40 msec.

TABLE I: Dephosphorylation of [^{32}P]Phospho-NDP-kinase.[a]

Expt	Reaction Time (msec)	Phosphate Acceptor	% of [^{32}P]-Phosphate Released
1	9.4	1 mM ADP	83
2	8.4	1 mM ADP	85
3	9.8	1 mM ADP 8.5 mM $MgCl_2$	42
4	10.4	1 mM ADP 10 mM EDTA	0

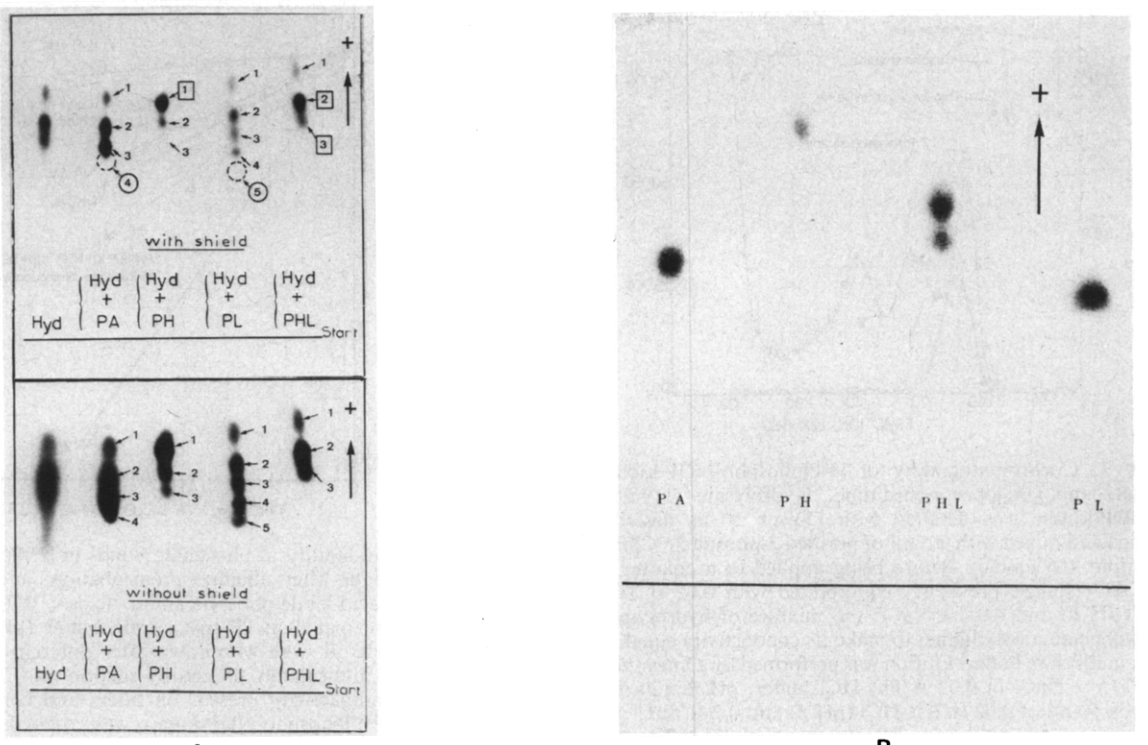
[a] [^{32}P]Phospho-NDP-kinase was purified as described in Results. Enzyme solution (1.8 ml; 17 × 10^3 dpm/ml) was mixed with an equal volume of ADP in 0.1 M phosphate buffer (pH 7) in the presence or in the absence of $MgCl_2$ in the rapid-flow apparatus and expelled into 2 ml of a cold solution of 3.75% trichloroacetic acid. The volume of medium expelled from the apparatus was between 0.44 and 0.55 ml, and the final pH of the mixture was 2.5. Two aliquots of 1 ml were immediately filtered in the cold on a Millipore membrane (0.22 μ, 13 mm diameter) which retained all the NDP-kinase. After three rapid washings with 0.25 ml of a cold mixture of 30% trichloroacetic acid–0.1 M phosphate buffer (pH 7)–water (4:20:16) at pH 2, the radioactivity of the Millipore membrane was measured in a gas-flow counter. Controls without phosphate acceptor were carried out to evaluate spontaneous dephosphorylation which ranged from 25 to 30%; the values given in the table have been corrected by subtraction of the control. Between 90 and 95% of ^{32}P present in [^{32}P]phospho-NDP-kinase was recovered in [^{32}P]ATP in the presence of ADP.

kinase was carried out by coelectrophoresis or cochromatography with alkali-stable phospho [^{14}C]amino acids, as described in Figures 6 and 7.

The results of a coelectrophoresis experiment are illustrated in Figure 6A by autoradiograms corresponding to X-ray films exposed either to both ^{32}P + ^{14}C or ^{32}P alone. Only spots corresponding to 3-phospho[^{14}C]histidine and N^{ϵ}-phospho[^{14}C]hydroxylysine were found to coincide with spots containing the ^{32}P radioactivity, suggesting a preliminary identification of the ^{32}P spots with 3-[^{32}P]phosphohistidine and N^{ϵ}-[^{32}P]phosphohydroxylysine. It must added that the ^{32}P spots did not correspond to [^{32}P]phosphopeptides since no difference in electrophoresis could be detected after treatment of the hydrolysate by trypsin, carboxypeptidases, A and B, and leucine aminopeptidase.

An increased ^{32}P radioactivity could be noticed on the autoradiogram (Figure 6A, upper part) at the level of 3-phosphohistidine (spot 1, track 3), when the [^{32}P]phospho-NDP-kinase hydrolysate was coelectrophoresed with 3-phospho-[^{14}C]histidine. This was accompanied by a lowering of the amount of less anionic ^{32}P compounds, located under the 3-phosphohistidine spot. Clearly, coelectrophoresis of the [^{32}P]-phospho-NDP-kinase hydrolysate and 3-phospho[^{14}C]histidine resulted in dragging some unknown, slowly moving, ^{32}P compound(s) to the level of 3-phosphohistidine. The 1-[^{32}P]phosphohistidine is the most liable candidate to this dragging effect. In fact, as shown by Hultquist (1968), 1-phosphohistidine, a less anionic compound than 3-phosphohistidine, therefore lagging behind 3-phosphohistidine in electrophoresis, is spontaneously and readily converted to 3-phosphohistidine whereas the reverse reaction is much slower. Based on Hultquist's work and assuming that the [^{32}P]phospho-NDP-kinase hydrolysate contained 1-[^{32}P]-phosphohistidine beside 3-[^{32}P]phosphohistidine, one must expect that mixing the [^{32}P]hydrolysate with a relatively large amount of 3-phospho[^{14}C]histidine (as it occurs in electrophoresis run) will lead, through transphosphorylation reactions, to the accumulation of 3-[^{32}P]phosphohistidine. The above rationale brings logical support in favor of the occurence of 1-phosphohistidine in phospho-NDP-kinase in spite of lack of direct chemical evidence.

Cochromatography of a ^{32}P-labeled NDP-kinase hydrolysate with 3-phospho[^{14}C]histidine on DEAE-cellulose column resulted in only one peak (peak I, Figure 7) coincident with 3-phospho[^{14}C]histidine. Two unidentified peaks (II and III) could be ascribed to undegraded phosphopeptides In contrast with coelectrophoresis data, N^{ϵ}-[^{32}P]phosphohydroxylysine could not be identified by cochromatography on DEAE-cellulose. Furthermore, there was no coincidence between the ^{32}P peaks and ^{14}C peaks corresponding to N^{ϵ}-phospho[^{14}C]lysine and N-phospho[^{14}C]arginine confirming the results of the coelectrophoresis experiment and therefore



A B

FIGURE 6: (A) Coelectrophoresis of hydrolysis products of [^{32}P]phospho-NDP-kinase with phospho [^{14}C]amino acids. The hydrolysate of [^{32}P]phospho-NDP-kinase was prepared and desalted as described in Methods. Twenty microliters of ^{32}P-labeled hydrolysate (about 1500 dpm) was spotted on Whatman No. 3MM paper alone or mixed with 10 μl of ^{14}C-labeled phosphorylated amino acid (about 8000 dpm for 3-phosphohistidine; 12,000 dpm for N^{ϵ}-phosphohydroxylysine, 13,000 dpm for N^{ϵ}-phospholysine, and 9000 dpm for N^{ϵ}-phosphoarginine). Electrophoresis was carried out at 50 V/cm for 50 min at 4° in a Pherograph apparatus, using a 0.09 M borate buffer (pH 8.5). After the electrophoretic run both sides of the electrophoretogram were exposed to two identical Kodak Kodirex films. One side of the paper was directly in contact with a film which was therefore exposed to both ^{32}P and ^{14}C radiations. A polyethylene sheet was interleaved between the other side of the electrophoretogram and the second film to shield the ^{14}C radiation and allow exposure only to ^{32}P. The two autoradiograms were compared for the localization of the ^{14}C and ^{32}P spots. Spots of ^{14}C and ^{32}P which appeared to coincide each other are designated by a square; those which were not coincident are designated by a circle. Location of the amino acids on the electrophoretogram was finally confirmed by means of ninhydrin. [^{32}P]Phosphate released during alkaline hydrolysis migrated toward the anode ahead of the phospho amino acids and is not shown in the photograph. (B) Electrophoresis of standard phospho [^{14}C]amino acids. Same conditions of electrophoresis as in part A.

excluding phospholysine and phosphoarginine as phosphorylated residues in beef heart cytosol NDP-kinase.

Acid Lability of Undegraded Phospho-NDP-kinase. The above mentioned data on identification of 3-phosphohistidine and possibly 1-phosphohistidine in degradation products of phospho-NDP-kinase were supported by a comparative study on the acid lability of the phosphate link(s) in undegraded phospho-NDP-kinase and in the four reference alkali-stable phospho amino acids. As shown in Figure 8, dephosphorylations of 3-phosphohistidine, N^{ϵ}-phospholysine and N^{ϵ}-phosphohydroxylysine, and *N*-phosphoarginine in citrate buffer (pH 3.6) at 25° are first-order processes. Among these four phospho amino acids, 3-phosphohistidine is the most stable and phosphoarginine the most labile. According to Hultquist (1968) the first-order rate constant for hydrolysis of 1-phosphohistidine, at pH 3.6, is about eight times as high as that of 3-phosphohistidine. Compared to data of Figure 8, the lability of 1-phosphohistidine would therefore be intermediate between that of phosphohydrolysine and that of phosphoarginine.

The rate of dephosphorylation of phospho-NDP-kinase *after a preincubation period of 1 hr at pH 12 and 25°* appears to be close to the rate of dephosphorylation of 3-phosphohistidine, in agreement with the identification of 3-phosphohistidine in alkaline hydrolysates of phospho-NDP-kinase. By contrast, when phospho-NDP-kinase was *immediately exposed to acid pH*, its rate of dephosphorylation was between that of phosphohydroxylysine and that of phosphoarginine, recalling the lability of 1-phosphohistidine as stated above. All these data, taken together, suggest that phosphate is bound to the N-1 of the imidazole ring of histidine in native phospho-NDP-kinase and that during the alkaline preincubation 1-phosphohistidine is isomerized into 3-phosphohistidine.

The pH-lability profile (Figure 9) expressed as the percentage of phosphate released from phospho-NDP-kinase also recalled that of 1-phosphohistidine as reported by Hultquist (1968) and provided further suggestive evidence for the presence of 1-phosphohistidine in phospho-NDP-kinase. An ambiguous trough observed in the region of pH 3 could reflect the presence of an acyl phosphate linkage. However this possibility was not consistent with the finding that hydroxylamine, a powerful reagent for the acid anhydride linkage, had no effect on enzyme activity.

Attempts to modify histidine residue(s) in NDP-kinase. Effect of diethyl pyrocarbonate and of photooxidation. Since histidine residue(s) of NDP-kinase can be phosphorylated by Mg-ATP, it is inferred that it is located in the active site of the enzyme. Under defined conditions, diethyl pyrocarbonate has been reported to combine specifically to histidine rseidues in proteins to give an ethoxycarbonyl derivative

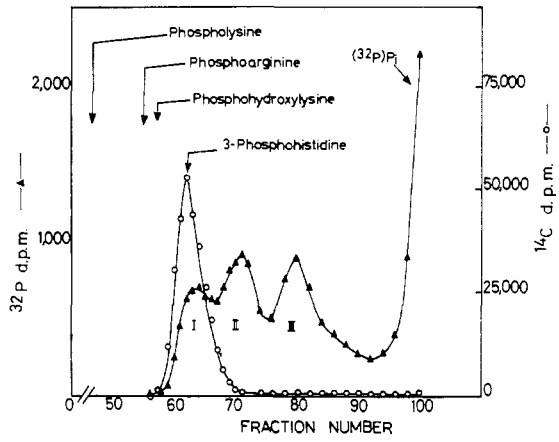


FIGURE 7: Cochromatography of [^{32}P]phospho-NDP-kinase hydrolysate and [^{14}C]phosphohistidine. Hydrolysate (1 ml) from [^{32}P]NDP-kinase was desalted with Dowex 50 as described in Methods and mixed with 2.5 ml of purified 3-phospho[^{14}C]histidine (7000 dpm, 0.6 μmole). Before being applied to a column of 5 g of DEAE-cellulose previously equilibrated with 0.02 M Tris-HCl buffer (pH 8) and 0.03 M NaCl, the mixture of hydrolysate and phosphohistidine was diluted to make its conductivity equal to that of the equilibrium buffer. Elution was performed by a linear gradient (0.03–0.15 M NaCl in 0.02 M Tris-HCl buffer, pH 8, 120 ml) followed by 50 ml of 0.02 M Tris-HCl (pH 8) and 0.2 M NaCl; 1.5-ml fractions were collected and aliquots were withdrawn for ^{32}P and ^{14}C determination by scintillation counting.

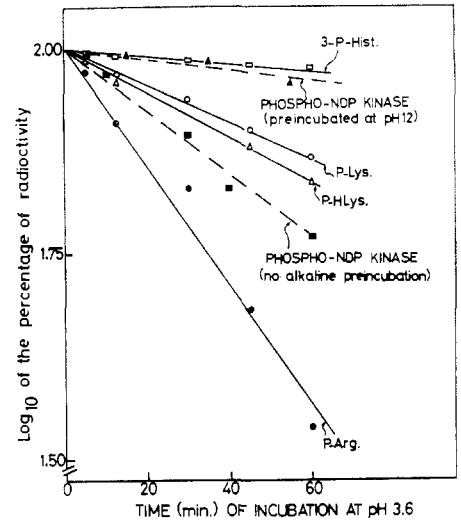


FIGURE 8: Acid lability of phosphate bonds in [^{32}P]phospho-NDP-kinase before or after alkaline preincubation, and of reference alkali-stable, acid-labile phospho amino acids. [^{32}P]Phospho-NDP-kinase was incubated in 20 mM citrate buffer (pH 3.6) at 25°. Aliquots of 150 μl were withdrawn after different periods of incubation and immediately filtered by suction on 0.22 μ Millipore filters. The radioactivity present on filters and corresponding to undegraded [^{32}P]phospho-NDP-kinase was counted (*cf.* Figure 5). Another test was carried out with [^{32}P]phospho-NDP-kinase after alkaline preincubation. [^{32}P]Phospho-NDP-kinase was first brought to pH 12–13 by addition of 0.1 N KOH and incubated at 25° for about 1 hr; then the pH was shifted to 3.6 by addition of citric acid. Incubation and withdrawing of samples were carried out as above. The acid lability of 3-phospho[^{14}C]histidine, N^{ϵ}-phospho-[^{14}C]lysine, N^{ϵ}-phospho[^{14}C]hydroxylysine, and *N*-phospho[^{14}C]-arginine was tested in parallel incubations (final concentration 0.5 mM) at pH 3.6 and 25° in 20 mM citrate buffer. Aliquots (30 μl) were withdrawn after different incubation periods and spotted on Whatman No. 3MM paper. The free [^{14}C]amino acids obtained by dephosphorylation were separated from the remaining phospho-[^{14}C]amino acids by electrophoresis, in a 0.09 M borate buffer (pH 8.5) at 50 V/cm for 50 min at 4°. After autoradiography, the areas of the electrophoretogram containing the radioactivity were located, cut out, and counted.

(Pradel and Kassab, 1968). Hydroxylamine selectively removes the ethoxycarbonyl group from imidazole (Melchior and Fahrney, 1970). A histidine residue at the active site of an enzyme can therefore be identified by the inhibitory effect of diethyl pyrocarbonate on the enzyme reaction and the reversal of this inhibition by hydroxylamine (Thomé-Beau *et al.*, 1971). Treatment of beef heart cytosol NDP-kinase by 1 mM diethyl pyrocarbonate at neutral pH for 5 min at 20° under conditions specified by Pradel and Kassab (1968) led to 80% inactivation, partly reversed (about 50%) by hydroxylamine. This result provided circumstantial support for a role of histidine at the active site of NDP-kinase.

Since histidine is liable to be photooxidized in the presence of a sensitizing dye, inactivation of NDP-kinase by photooxidation was taken as another approach to verify whether histidine played a role in NDP-kinase catalysis. Histidine photooxidation is a first-order and pH-dependent process (Westhead, 1965). This pH dependence distinguishes histidine from other photooxidizable amino acids, namely cysteine, methionine, tryptophan, and tyrosine. For this purpose the NDP-kinase photooxidation was carried out at various pH ranging from 5 to 9. Rose Bengal was chosen instead of Methylene Blue because its anionic structure is more consistent with an approach of the active site of the enzyme. Beef heart cytosol NDP-kinase was readily photooxidizable. But the rate of photooxidation was of the same order at pH 9 and 5. Absence of pH effect (from pH 6 to 8.5) has previously been noticed for photooxidation of rabbit muscle aldolase in spite of the fact that histidine residues are involved in catalysis (Hoffee *et al.*, 1967). To explain this abnormal behavior, Hoffee *et al.* have postulated that histidine residues at the active site of aldolase are unprotonated even at pH 6, due to a lipophilic environment. A similar explanation may hold in the case of NDP-kinase; besides, the rapid spontaneous inactivation of this enzyme below pH 5.5 may interfere with the pH dependence of histidine photooxidation.

Inactivation of NDP-kinase by photooxidation was about three times more rapid in the presence than in the absence of EDTA. At pH 8, in the presence of EDTA, the rate of photooxidation was first order and markedly decreased by addition of ADP or ATP (Figure 10). The methylene analog of ATP, AOPOPCP, which is not a substrate for NDP-kinase, did not protect the enzyme against photooxidation. Protection against photooxidation in a Mg^{2+} supplemented medium was also afforded by ADP and ATP. The observation that free ADP or free ATP (EDTA-supplemented medium) protect NDP-kinase against photooxidation is quite surprising in view of the kinetic demonstration that Mg-ADP and Mg-ATP bind to the enzyme preferentially to the free nucleotides; however occurence of unstable dead-end complexes between NDP-kinase and free ATP or free ADP, is not excluded.

Discussion

Isolation and characterization of a stable phospho derivative of beef heart cytosol NDP-kinase, as reported here, is in good agreement with the demonstration of the Ping-Pong kinetics catalyzed by this enzyme. An ATP-dependent phosphorylation has also been demonstrated for NDP-kinases

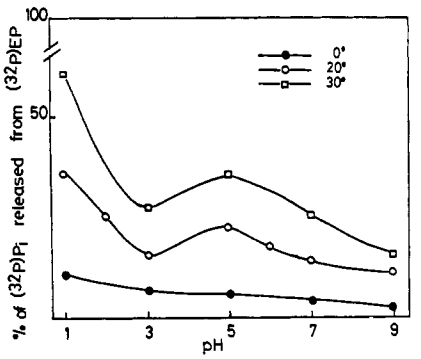


FIGURE 9: pH-lability profile of phospho-NDP-kinase. Samples (0.1 ml) of [^{32}P]phospho-NDP-kinase collected from Bio-Gel P4 in 0.1 M phosphate buffer (pH 7) were distributed in several tubes and the pH of each sample was adjusted to the desired value with 1 N HCl (pH 1–2) or 1 M citric acid (pH 3–6) or 1 N NaOH (pH 8–9). After 30-min incubation 50 μl of each fraction was withdrawn and spotted on Whatman No. 3MM paper for electrophoretic separation of [^{32}P]phosphate. After autoradiography to locate the radioactive areas of the electrophoretograms, the spots containing the [^{32}P]inorganic phosphate were cut out and counted.

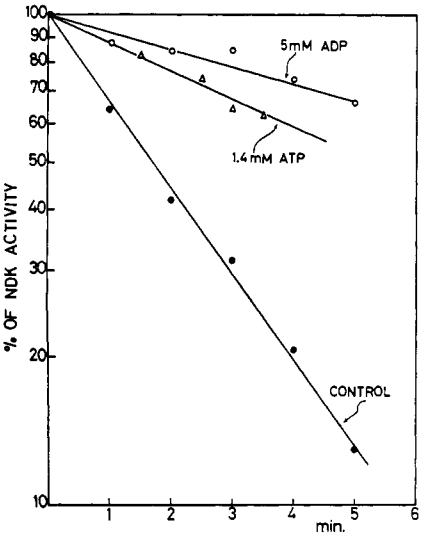


FIGURE 10: Inactivation of NDP-kinase by photooxidation. Protection by ADP and ATP. NDP-kinase (1 ml; 7.2 μg/ml) in phosphate buffer (0.1 M, pH 8) was irradiated, as described in Methods, in the presence of 10^{-5} M Rose Bengal and 10^{-4} M EDTA; where indicated 1.4 mM ATP or 5 mM ADP were added to the incubation medium. The results are expressed as the percentage of enzyme activity remaining after photooxidation for different periods of time as compared to that of a control kept in the dark for the same time.

extracted from erythrocytes (Mourad and Parks, 1965), Jerusalem artichoke mitochondria (Norman *et al.*, 1965), beef heart mitochondria (Colomb *et al.*, 1966), yeast (Garces and Cleland, 1969), beef liver mitochondria (Pedersen, 1968), beef liver (Wälinder, 1968), Baker's yeast (Edlund *et al.*, 1969), and pea seeds (Edlund, 1971). Thus, NDP-kinase phosphorylation may be considered as a general mechanism in which NDP-kinase functions as a phosphate carrier between a nucleoside triphosphate and a nucleoside diphosphate.

Evidence has been presented in this paper for phosphorylation of histidine residue(s) in beef heart cytosol NDP-kinase. 3-[^{32}P]Phosphohistidine was characterized in the products of alkaline hydrolysis of [^{32}P]phospho-NDP-kinase by comigration with 3-phospho[^{14}C]histidine in paper electrophoresis and in DEAE-cellulose chromatography. A small amount of 1-[^{32}P]phosphohistidine was also present in the hydrolysate. In coelectrophoresis with 3-phospho[^{14}C]histidine the slower 1-[^{32}P]phosphohistidine moved at the level of 3-phosphohistidine, a "dragging" effect likely to be due to the known migration of phosphate between N_1 and N_3 of the imidazole ring of histidine (Hultquist, 1968). Another criterion for the identification of 1- and 3-phosphohistidine was the acid lability of the phosphate bond(s) in undegraded phospho-NDP-kinase. Quite unexpectedly, the rate of phosphate release at pH 3.6 was found dependent on preincubation in alkaline medium (pH 12, 25°, 1 hr). Without preincubation, the acid lability of the phosphate bond in phospho-NDP-kinase was virtually similar to that of the phosphate bond in 1-phosphohistidine; after alkaline preincubation, it was comparable to that of 3-phosphohistidine. These data may be explained in terms of migration of phosphate from N_1 to N_3 of histidine (Hultquist, 1968) during the alkaline preincubation of phospho-NDP-kinase. They suggest that most of, if not all, the phosphohistidine present in native phospho-NDP-kinase is 1-phosphohistidine, which is in agreement with the demonstration of 1-phosphohistidine, in phospho-NDP-kinases from bovine liver (Wälinder, 1968), human erythrocytes (Wälinder *et al.*, 1968) baker's yeast (Edlund *et al.*, 1969), pea seeds (Edlund, 1971).

Other supporting evidence for the presence of phosphohistidine in beef heart cytosol phospho-NDP-kinase comes from inactivation studies using diethyl pyrocarbonate, a protein histidyl reagent reported to inactivate several phosphotransferases by binding to a critical histidine residue (Pradel and Kassab, 1968).

Alkali-stable phospho amino acids other than phosphohistidine, namely phosphoarginine, N^{ϵ}-phosphohydroxylysine, and N^{ϵ}-phospholysine, could not fit the different criteria satisfied by phosphohistidine and were therefore considered to be absent in beef heart cytosol phospho-NDP-kinase. As far as phospholysine is concerned, this is at variance with the NDP-kinases from human erythrocytes and bovine liver (Wälinder, 1968), and from pea seeds (Edlund, 1971).

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Kinetics and the Mechanism of Action of Adenosine Aminohydrolase†

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ABSTRACT: The kinetics of calf duodenum adenosine aminohydrolase were investigated. Inosine was found to be a competitive inhibitor and ammonia a noncompetitive inhibitor. These results are consistent with an ordered release of products with ammonia being the first. Comparison of certain kinetic constants to K_{eq} suggest that the minimal mechanism is ordered uni-bi in which the isomerization of an enzyme–product complex plays a significant role. Methanol gave complex inhibition kinetics, which could be interpreted in terms of mixed dead-end and alternate product inhibition and was consistent with a crypto Ping-Pong mechanism in which the hydrolytic step occurs after the rate-limiting release of ammonia. A single cationic group with a pK_a of 4.8 was found to control the rate-limiting step. Two groups with pK_a of 5.7 and 8.7 were shown to be essential for binding of the substrate, with the latter being equated with a thiol residue on the basis of inactivation by *p*-chloromercuribenzoate. These data are consistent with a previously proposed mechanism in which a tetrahedral thiopurinyl-enzyme compound is a compulsory intermediate.

Adenosine aminohydrolase has a widespread distribution in animal tissues (Conway and Cooke, 1939; Makarewicz and Zydowo, 1962) and in all species the duodenum and spleen are the richest sources (Brady and O'Donovan, 1965). A number of these enzymes have been purified, including those from calf duodenum (Brady and O'Connell, 1960; Chilson and Fisher 1963) and chicken duodenum (Hoagland and Fisher, 1967).

Although much work has been done on the specificity of the calf duodenum enzyme, especially by Schaeffer and his coworkers (Schaeffer *et al.*, 1964; Schaeffer and Bhargava, 1965; Schaeffer and Vogel, 1965), little is known about the mechanism of action. In common with most other aminohydrolases this enzyme appears to require a thiol residue for full activity (Ronca *et al.*, 1967; Pfrogner, 1967) and although fairly specific for the purine ribonucleoside moiety (Frederiksen, 1966) it is relatively nonspecific in regard to the substituent on the C_6 of the purine ring (Chassy and Suhadolnik, 1967; Baer and Drummond, 1966). In 1969 Bolen and Fisher proposed that the chicken duodenal enzyme operated by an ordered sequential mechanism. They based this conclusion on the irreversibility of the reaction and the observation that log K_m/V_m was a linear function of the surface tension in a wide variety of solvents. In contrast to this Wolfenden (1966), using the nonspecific adenosine aminohydrolase from *Aspergillus oryzae*, observed a constant maximum velocity for a series of C_6-substituted purine ribonucleosides and proposed a Ping-Pong or substitution mechanism in which a purinyl ribonucleoside–enzyme compound is formed as a compulsory intermediate. It was considered that a kinetic approach through product and alternate product inhibition (Cleland, 1963a) and evaluation of the kinetic constants (Hsu *et al.*, 1966) might resolve certain aspects concerning the mechanism of adenosine aminohydrolase.

Materials

Adenosine, 2′-deoxyadenosine, inosine, 6-methylaminopurine ribonucleoside, 6-methoxypurine ribonucleoside, 6-hydrazinopurine ribonucleoside, 6-chloropurine ribonucleoside, and *p*-chloromercuribenzoate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Diisopropyl fluorophosphate

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