DETECTION OF PHOSPHOHISTIDINE IN NUCLEOSIDE DIPHOSPHOKINASE ISOLATED FROM JERUSALEM ARTICHOKE MITOCHONDRIA*

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The work of Boyer et al. (1962), Mitchell et al. (1964), and Lindberg et al. (1965) has demonstrated the incorporation of ^{32}Pi or $AT^{32}P$ into phosphohistidine found in mammalian mitochondria under conditions indicating its probable participation in phosphorylation reactions. The presence of phosphohistidine in succinate thiokinase (ST-kinase) obtained from E. coli was also shown by Kreil and Boyer (1964), and more recently by Wedding et al. (1965) in a highly purified succinate thiokinase isolated from mitochondria of tubers of the Jerusalem artichoke (Helianthus tuberosus).

A major interfering enzymatic activity present during the isolation of the artichoke mitochondrial ST-kinase was that corresponding to a nucleoside diphosphokinase (NDP-kinase); one catalyzing the following reaction: $n'TP + nDP \rightleftarrows n'DP + nTP$

A final chromatographic step on a DEAE cellulose column utilizing a stepwise elution with $(NH_4)(H_2PO_4)$ buffer at pH 8.0, yielded two protein peaks; one containing only ST-kinase (specific activity 24.5 μmolar units/mg of protein[1]), and one with NDP-kinase (specific activity 3.3

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[1]A unit is defined as the μmoles of succinyl CoA produced per minute at 27° in a reaction mixture consisting of 0.2 μmoles CoA, 50 μmoles succinate, 0.6 μmoles ATP, 10 μmoles $MgCl_2$, and 100 μmoles Tris, pH 7.4 in a total volume of 1.3 ml, read at 232 mμ with a 0.3 cm light path.

μmolar units/mg of protein[2]) completely devoid of ST-kinase activity. The details of the ST-kinase isolation have been previously presented (Palmer and Wedding, 1965).

While studying the labeling of the ST-kinase from $^{32}P_i$ and $AT^{32}P$, it became pertinent to examine the labeling patterns of mixtures of the ST-kinase and NDP-kinase. Experiments of this nature revealed that NDP-kinase also had the capability of becoming labeled from $AT^{32}P$. These experiments are in agreement with the recent report by Mourad and Parks (1965) that NDP-kinase (isolated from human erythrocytes) has a phosphorylated enzyme as the reactive intermediate.

The demonstration of the presence of the ^{32}P-NDP-kinase obtained from Jerusalem artichoke mitochondria is shown in figure 1 and table 1. When

Table 1

Demonstration of ^{32}P-NDP-kinase

Treatment	Percent of ^{32}P in protein fraction	
	Sephadex column	Phenol extraction
Incubated sample	0.016	0.020
Control ($AT^{32}P$ added after EDTA)	0.001	0.003

NDP-kinase (0.9 units) was incubated in 0.5 ml with Tris-Cl, pH 7.2, 10 μmoles; $MgCl_2$, 1.0 μmole; and $AT^{32}P$, 0.2 μmoles containing 6.2 X 10^6 cpm. After 60 seconds 0.75 ml of 0.25 M EDTA, pH 7.2, was added. The sample was divided into two equal aliquots. One was chromatographed on a Sephadex-G-25 column as in figure 1. 2.0 ml of liquid phenol and 5.0 mg of carrier bovine albumin was added to the second aliquot and the ^{32}P-protein determined by the extraction procedure of Bieber et al. (1964).

[2]A unit is defined as the μmoles of TPNH produced per minute at 27° in a reaction mixture consisting of 3 μmoles TPN, 0.6 μmoles GTP, 0.3 μmoles ADP 5 μmoles glucose, 5 μmoles $MgCl_2$, 2 units hexokinase, 0.5 units glucose-6-phosphate dehydrogenase and 100 μmoles Tris, pH 7.8 in a total volume of 1.3 ml, read at 340 mμ with a 0.3 cm light path.

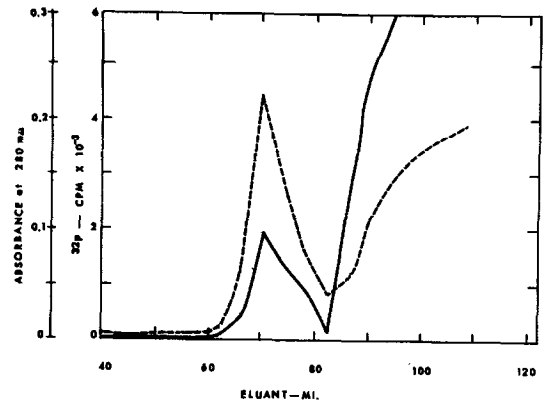

Figure 1: Separation of ^{32}P-labeled NDP-kinase from AT^{32}P on Sephadex-G-25. The reaction mixture in 2.1 ml consisted of: Tris-Cl, pH 7.2, 100 μmoles; $MgCl_2$, 10 μmoles; ATP. 1.2 μmoles containing 35.5 X 10^3 cpm; and NDP-kinase 9.8 units, specific activity 3.0 μmolar units/mg protein. The reaction after incubation at 27°C for 4 minutes was terminated by the addition of 0.4 ml of 0.25 M EDTA, pH 7.2. The sample was immediately placed on a 40 X 2.5 cm Sephadex-G-25 column and eluted with 0.10 M Tris-Cl, pH 7.2, at a flow rate of 1.0 ml/min; - - - - absorbance at 280 mμ. ———— cpm/tube. 0.5 ml fractions were collected.

Table 2

Transfer of ^{32}P from NDP-kinase to Diphosphonucleotides

Acceptor	Percent of Total Counts Incubated Berenbloom & Chain Distribution		
	Phenol Layer (Protein)	Lower Layer (Nucleotide)	Upper Layer (P_i)
None	100	0	0
ADP	4	90	6
UDP	3	92	5
GDP	11	81	8

^{32}P-NDP-kinase containing approximately 900 cpm and 0.6 units was incubated in 2.2 ml with $MgCl_2$, 10 μmoles; Tris-Cl, pH 7.2, 30 μmoles; and acceptor diphosphonucleotide, 2.0 μmoles, where indicated. After 4 minutes, the incubation was terminated by the addition of 2.0 ml of liquid phenol. After thorough mixing and centrifugation, the ^{32}P in the aqueous layer was partitioned by the Lindberg and Ernster (1956) modification of the Berenbloom and Chain (1938) assay, which extracts P_i as the molybdate complex into the upper, isobutanol-benzene layer, leaving nucleotides in the lower layer. The phenol layer was washed four times with 5.0 ml of 0.01 M P_i - 0.01 M EDTA, pH 7.2 and then the radioactivity content determined.

the NDP-kinase is incubated with Mg^{++} and $AT^{32}P$ the ^{32}P-protein can be detected by either the phenol extraction procedure of Bieber et al. (1964) or by chromatography on Sephadex-G-25 columns. Comparable results are obtained by either assay. Little, if any, enzymatic activity is lost by chromatography on Sephadex columns.

Several acceptor diphosphonucleosides are capable of removing the ^{32}P-moiety of the ^{32}P-NDP-kinase, as isolated from the Sephadex-G-25 columns, in a manner characteristic of a nucleoside diphosphokinase-catalyzed reaction (table 2). After reincubation of the ^{32}P-protein with acceptor nDP, and addition of phenol, the presence of ^{32}Pi or nucleotide-^{32}P was determined by the Lindberg and Ernster (1956) modification of the Berenblom and Chain (1938) distribution procedure. Nucleotide-^{32}P was assumed to be that radioactivity remaining in the lower layer, while ^{32}Pi was that radioactivity extracted into the upper layer as the isobutanol-benzene soluble molybdate complex. As shown in table 2, ADP, GDP and UDP appear to function equally effectively in removing the ^{32}P-moiety from the ^{32}P-NDP-kinase. However, when no acceptor nDP was present during the incubation, the ^{32}P-moiety was retained with the protein in the phenol layer.

To eliminate the possibility that the data presented in tables 1 and 2 and figure 1 resulted from a binding of $AT^{32}P$ to the enzyme rather than transfer of the γ-^{32}P to an acceptor moiety on the protein, the acid and alkaline labilities of the ^{32}P-protein as isolated from the Sephadex column were determined. The results, shown in table 3, leave little doubt as to the difference in hydrolytic behavior between the ^{32}P-NDP-kinase and $AT^{32}P$. The alkaline stability and marked acid lability are characteristic of phosphohistidine. A first order rate constant for the acid catalyzed hydrolysis of ^{32}P-NDP-kinase at pH 3.8, 60° of 0.025 - 0.035 per minute was obtained. This is in good agreement with that of authentic phosphohistidine[3].

As a means of confirming the tentative identification of the presence

[3]Personal communication, Prof. P. D. Boyer.

Table 3

Acid Lability and Alkaline Stability

of ^{32}P-NDP-kinase

Experimental Treatment	pH	Percent of Total Counts Released as $^{32}P_i$	
		^{32}P-protein	AT^{32}P
1 minute, 100°	1.5	100	5
2 minutes, 100°	1.5	100	10
4 minutes, 60°	3.8	7.2	0.3
30 minutes, 60°	3.8	33.8	1.0
4 minutes, 60°	10.2	0	†
30 minutes, 60°	10.2	0	†

^{32}P-NDP-kinase or AT^{32}P containing approximately 2000 cpm in 0.014 M LiCl was adjusted to pH 3.8 with glacial acetic acid, to pH 1.5 with 0.30 M trichloroacetic acid, and pH 10.2 with 0.1 M NaOH. After heating under the stated conditions the samples were rapidly cooled to 2-4° C, and the $^{32}P_i$ measured by the Lindberg and Ernster (1956) modification of the Berenbloom and Chain (1938) distribution method.

†Under alkaline condtions ATP is rapidly degraded to inorganic pyrophosphate and AMP. (Dawson et al. 1959).

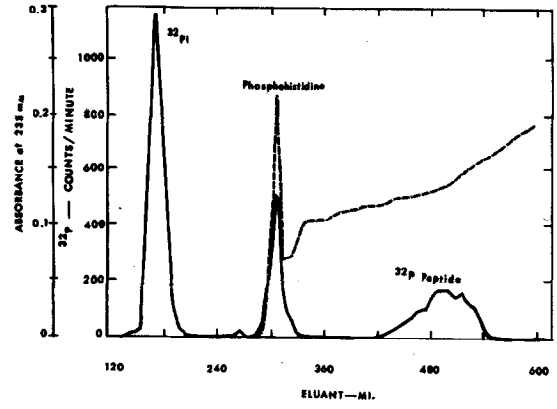

Figure 2: Cochromatography of alkaline hydrolysate of ^{32}P-NDP-kinase with authentic phosphohistidine. The ^{32}P-protein sample from figure1 was digested with 10 mg of synthetic phosphohistidine in 3.0 M NaOH at 110° C for 90 minutes. After adjusting the pH of the hydrolysate to 10.8 with Dowex-50, H^+, the sample was chromatographed on Dowex-1-(OH) according to the method of Boyer et al, (1962) using a linearly increasing gradient of $NaHCO_3$-CO_3, pH 8.5.- - - - absorbancy at 232 mμ; ———— cpm/tube.

of phosphohistidine in NDP-kinase, the radioactive protein of figure 1 was mixed with authentic phosphohistidine[4] and subjected to alkaline hydrolysis and chromatographed by the procedure of Kreil and Boyer (1964) to give the pattern shown in figure 2. One peak of radioactivity coincides with the absorbance peak of authentic phosphohistidine. Two other peaks with ^{32}P activity were found. One behaves as ^{32}Pi in the Berenbloom and Chain distribution method, and the other probably represents incompletely hydrolyzed peptides containing phosphohistidine (Kreil and Boyer, 1964), although no attempt was made to confirm this identification.

Although the results strongly suggest the involvement of phosphohistidine as an essential participant in the NDP-kinase catalyzed reaction, the homogeneity of the NDP-kinase has not been rigidily established. Thus even though the NDP-kinase is completely free of ST-kinase, the close association of two or more other proteins remains a possibility.

This demonstration of phosphohistidine in nucleosidediphosphokinase lends support to the ubiquitous role of phosphohistidine as a phosphoenzyme intermediate (Kreil and Boyer, 1964, Kundig et al., 1964). The fact that this is the second enzyme found in the mitochondria which contains phosphohistidine confirms the suggestions of Lindberg et al. (1965) who obtained indirect evidence for mitochondrial sources of bound phosphohistidine other than the succinate thiokinase system. This finding also emphasizes the difficulty of explicitly defining the role of bound phosphohistidine in mitochondrial phosphorylation reactions with the presently available techniques.

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[4]Kindly provided by Prof. P. D. Boyer.

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