MOLECULAR SPECIES OF A SOLUBLE NUCLEOSIDE DIPHOSPHOKINASE RELATED TO THE Na^+,K^+-ATPase.

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Soluble nucleosidediphosphokinase (NDK) activity is prepared from purified kidney membranes rich in Na^+,K^+-ATPase. The soluble NDK differs from the bound activity by the lack of sensitivity to Na^+, K^+ and ouabain. Sucrose gradient centrifugation of the soluble NDK shows the presence of three major species with molecular weights of 21,000, 92,000 and 138,000. Increasing the urea concentration in the sucrose gradient centrifugation decreases the proportion of the enzyme in the heavier form. This effect of urea is reversible. These data suggest that the higher molecular weight forms are tetramers and hexamers of the 21,000 dalton form of the soluble NDK.

The Na^+-stimulated phosphorylation of Na^+,K^+-ATPase preparations by γ-$AT^{32}P$ is one of the initial steps of the ATPase system [for a review, see Albers (1)]. Fahn et al. (2) have shown that the Na^+-stimulated exchange of ADP to ATP activity also represents this initial step. This enzymatic activity catalyzes the reaction

$$ATP + E \underset{<}{\overset{Na^+,Mg^{2+}}{\longrightarrow}} E \sim P + ADP \qquad (1)$$

and is therefore referred to as nucleosidediphosphokinase (NDK).

After extraction of pig kidney membranes with NaI we observe soluble NDK activity. It differs from the membrane bound NDK, in that it is neither stimulated by Na^+ nor is it inhibited by K^+ or ouabain. In all other properties tested the two NDK activities behave in similar ways. The

soluble NDK fraction shows different molecular species of 21,000, 92,000 and 138,000 daltons.

Membranes are prepared from pig kidney and solubilized with 2 M NaI as previously described (3). The soluble fraction is further purified by a 25-45% saturated $(NH_4)_2SO_4$ fractionation which removes ATPase and phosphatase activities. NDK activity is measured either by labeling of enzyme with γ-AT^{32}P, carried out as described by Post *et al.* (4), or by the ^{14}C-ADP to ATP exchange reaction as described (3). Molecular forms are measured by sucrose gradient centrifugation according to Martin and Ames (5).

Similarities and differences between the membrane bound and soluble NDK activities are shown in Table I. Both activities require Mg^{2+} and are inhibited to the same extent by the same concentration of either Ca^{2+} or p-chloromercuribenzoate (pCMB). The rate of formation of the phosphorylated protein is very fast (exp. #5 and 6). In both NDK activities the complex formed is readily chased by addition of cold ATP. Only the phosphate from ATP reacts with the enzyme. The complex is not soluble in chloroform-ethanol, indicating that it is not a lipid. In both systems, the bound phosphate is released by NH_2OH. Our results on the properties of the membrane bound NDK activity are in keeping with those reported by others (4, 6, 7, 8).

The last three experiments in Table I indicate the differences between the membrane bound and the soluble NDK. The Na^+ stimulation seen with the membrane bound fraction is lost during solubilization of the enzyme. The soluble NDK is insensitive to K^+ or ouabain (a specific inhibitor of the Na^+,K^+-ATPase).

TABLE I

CONDITIONS OF PHOSPHORYLATION WITH MEMBRANE BOUND AND SOLUBLE NUCLEOSIDE DIPHOSPHOKINASE

Enzyme (0.5 mg protein) incubated in 100 mM tris-HCl, pH 7.5, 0.4 mM $MgCl_2$, 120 mM NaCl, 0.04 mM EGTA, 0.2 mM γ-$AT^{32}P$ for 10 sec at 0°C

		μμmoles ^{32}P bound/mg protein	
		Kidney Membranes	Soluble
1.	Complete system	116	15
2.	Complete system -$MgCl_2$	20	1.7
3.	Complete system + 1 mM $CaCl_2$	69 (42%)	9.1 (39%)
4.	Complete system + 0.1 mM pCMB	51 (56%)	5.4 (64%)
5.	Complete system, 60 sec incubation at 0°	116	22
6.	Complete system, 10 sec incubation at 37°	---	15
7.	Complete system, + chase with 2 mM ATP for 10 sec	6.9	< 1
8.	Complete system, -$AT^{32}P$ + ^{14}C-ATP	< 1	< 1
9.	Complete system, after chloroform-ethanol extraction	104	13
10.	Complete system, after incubation with 0.8 M NH_2OH	14	5.6
11.	Complete system, + 10 mM KCl	28	15
12.	Complete system, -NaCl	13	15
13.	Complete system, + 1 mM ouabain	75	14

Further evidence of the similarities of the protein-^{32}P complex in both systems is seen in Fig. 1 where the effect of pH on the chemical stability of the bound ^{32}P is shown. After the protein-^{32}P complex is suspended in buffers of varying pH for 20 minutes at 37°, the protein is reisolated and counted. The data is expressed as % of initially bound ^{32}P. It can be seen that in both systems the greatest stability is observed at pH 2. At higher or lower pH, the bound ^{32}P is less stable. The pH stability of the phosphorylated intermediate as shown in Fig. 1 is very similar to that reported by other investigators (6, 7, 8).

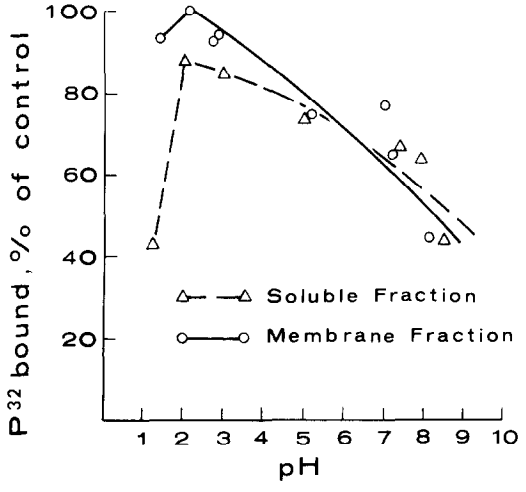

Fig. 1 - The effect of pH on the chemical stability of the phosphorylated protein. Fractions were labeled as described in Table I; see text for remaining details of the experiment.

The close catalytic and chemical relationships of the two NDK forms do not prove, but strongly support, the contention that the soluble form of the NDK is part of the Na^+ stimulated NDK of the Na^+,K^+-ATPase.

The molecular forms of the soluble NDK are determined by sucrose gradient centrifugation (Fig. 2). Three major

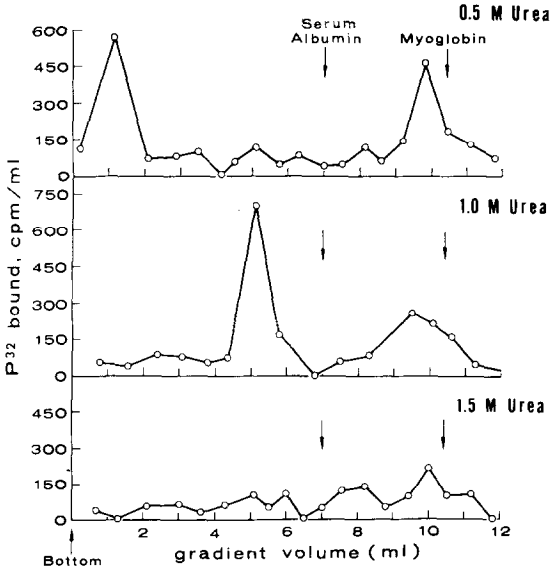

Fig. 2 - Sucrose gradient centrifugation of the soluble NDK. Centrifugation was for 36 hours at 40,000 rpm in a SW 41 rotor. Gradients were 5-20% sucrose in 20 mM tris (pH 7.5) - 0.5 mM mercaptoethanolamine and 0.5, 1.0 or 1.5 M urea. Fractions were labeled as described in Table I.

peaks of activity are evident. The molecular weights are determined from the positions of the markers shown, based on the assumption that the NDK is a lipid-free and globular protein. The molecular weights in the upper run correspond to 21,000 and 138,000, while the ones seen in the middle run are 21,000 and 92,000 respectively. The observed molecular weights are in keeping with the interpretation that they represent monomer, tetramer, and hexamer forms. The ratio of each component depends on the urea concentration in the sucrose gradient mixture of the centrifugation. At 0.5 M urea (upper run) the hexamer and monomer are the major forms, at 1.0 M urea (middle run), the tetramer and monomer are the major forms, and at 1.5 M urea (bottom run), the monomer is the only major peak. Low activity seen in the 1.5 M urea is due to urea inhibition both in the assay and during the

TABLE II

MOLECULAR WEIGHT OF SOLUBLE NUCLEOSIDE DIPHOSPHOKINASE ESTIMATED FROM SUCROSE GRADIENT CENTRIFUGATION

Molecular Weight from Peaks of Enzymatic Activity	
Assayed by Phosphorylation	Assayed by ^{14}C-ADP-ATP Exchange
20,760 ± 3,340 (5)	18-24-25,000
44,000 ± 5,200 (7)	40-41-55,000
67,500 ± 7,560 (4)	69,000
92,080 ± 6,180 (6)	91-92-100,000
116,750 ± 5,820 (6)	107-112-123,000
138,200 ± 9,230 (5)	136-138-155,000

Data in left column are mean ± standard deviation. Number of determinations are in parenthesis.

run. The effect of urea is therefore only qualitatively reversible. If the enzyme prepared in 1.5 M urea is subsequently dialyzed in 0.5 M urea and run on a gradient containing 0.5 M urea, the hexamer peak reappears. Table II shows averages of the observed molecular forms from different runs. The minor peaks of activity as seen in Fig. 2 have also been tabulated and they correspond well to dimer, trimer, pentamer forms. In several runs, the NDK was also determined by measuring the ^{14}C-ADP into ATP exchange activity. As one can see in Table II, the observed molecular weight for this activity falls within the range for the forms determined by the other assay method.

The soluble NDK shows three major molecular forms of average molecular weight 21,000, 98,000 and 138,000. The ratio of these different forms depends upon the urea concentration and is in keeping with the formation of tetramer and hexamer from the monomer fraction. The molecular weight of the tetramer is very similar to that reported for the protein-^{32}P complex of the Na^+,K^+-ATPase. Alexander and

Rodnight (9) have reported a molecular weight of 104,000 and Uesugi et al. (10) 94,000 daltons. Their values may indicate that the molecular form of the NDK in the Na^+,K^+-ATPase is a tetramer.

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