SOME ACYL PHOSPHATE-LIKE PROPERTIES OF P^{32}-LABELED SODIUM-POTASSIUM-ACTIVATED ADENOSINE TRIPHOSPHATASE

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In recent years there have been several studies reporting incorporation of phosphate moiety of ATP into Na^+-K^+-activated, so-called transport ATPase preparations (1, 2,3,6,13,14,17). It was suggested that a serine residue might be the binding site of the phosphate (7), while others consider that the acid lability of the complex (1,2) is difficult to be reconciled with this assumption (2,5). On the other hand, recent investigations indicate that an unspecified phosphate-protein complex may play a role in mitochondrial ion transportation (12). The complex may or may not be a phosphohistidine compound (11). In view of these results, characterization of the binding site of the phosphate in transport ATPase deserves further investigations.

We have obtained, using NaI-treated microsomal enzyme preparations (10), some results suggesting that the intermediate may consist of a single chemical species. Short accounts of them and a tentative characterization of the linkage will be presented here.

Materials and Methods:

The microsomal fraction from rabbit brain cortex was the enzyme source. The procedure of the enzyme preparation is described in the accompanying paper (10). In the following experiments, relatively large amounts of the enzyme protein were needed, and microsomes were treated with NaI in higher

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protein concentrations (10-20 mg/ml) than the values of 3-5 mg/ml in the standarized procedure. This situation tended to lower the specific activity of the preparation (60-110 μmoles/mg protein/hr. Cf. Table 1 in reference 10). However, they were active enough for the present work.

ATP^{32} was prepared enzymatically by incubating inorganic radioactive phosphate (Pi^{32}) with red cells (16), or synthesized chemically (9). In either case, the products were separated and purified through charcoal (Norit A) and anion exchange resin (Dowex-1, Cl^--form) columns.

For labeling the ATPase preparation, it was incubated in the reaction mixture (total volume 1 or 2 ml) containing: 2-4 mg of the protein/ml; ATP^{32}(final concentrations 4-9 x 10^{-5}M; specific activity 1-20 μc/μmole); 5mM $MgCl_2$; 40mM Tris-HCl buffer (pH 7.5); and 140mM NaCl or 14mM KCl (referred to as Na- and K-system, respectively). The mixture was incubated at 0° for a short period, which was usually 5 seconds. The reaction was stopped by adding equal volume of 4% sodium dodecyl sulfate (SDS) solution, or 3 volumes of 4% perchloric acid (PCA) to the reaction mixture. Other details are specified in the legends. In several experiments, the KCl concentration was 140mM instead of 14mM. No essential differences in the results were found between low and high K concentrations.

The protein precipitated by PCA was washed quickly 4 times with cold 0.5N PCA containing 10^{-4}M Pi and 10^{-4}M ATP as carriers, and counted in a low-background gas-flow counter. When the reaction was stopped by SDS, PCA was not used (see the legend for Table 1).

Results and Discussion:

a) Comparison of Conditions for the Labeling Experiments

Incorporation of P^{32} into the ATPase preparation was much higher in Na-systems. In a typical experiment, a value of 0.6 μmoles P/g protein was found for Na-systems; while only 0.074 μmoles P/g for K-system. Incorporation into several different ATPase preparations ranged between 0.1 and 1 μmole P/g protein in Na-system. Opposite effects of Na and K have also been recognized by other authors (1, 2,17) and led them to propose reaction schemes which can be summarized as follows:

$$E + ATP \overset{Mg^{++}}{\rightleftharpoons} E_1P \overset{Na^{+}}{\rightleftharpoons} E_2P \overset{K^{+}}{\rightleftharpoons} E + Pi \quad (I)$$

P^{32}-labeled protein was also obtained at neutral pH when the reaction was stopped by adding sodium dodecyl sulfate (SDS) solution to a final concentration of 2%. The protein was soluble in the detergent solution and easily separated from the remaining ATP^{32} and Pi^{32} by passage through a Sephadex G-25 column. Sodium and potassium ions again showed their contrasting effects (Table 1). Thus, P^{32}-proteins obtained under different conditions were affected in the same way by the presence of alkali ions. This fact reinforces the argument that the labeled protein is not an artifact but the true intermediate.

SDS was not used in the following work, since less incorporation was obtained by use of this reagent (e.g., after 5 and 15 second incubation, 0.17 and 0.16 μmoles P/g protein were found when the reaction was stopped with PCA; while the corresponding values with SDS were 0.045 and 0.072).

Table 1. Effects of Na and K on P^{32} incorporation into ATPase preparation

Na (mM)	K (mM)	Incubation (seconds)	P^{32} incorporated (μmole/g protein)
140	0	5	0.107
0	14	5	0.012
140	14	5	0.018
140	0	0	0.002

The labeling was stopped by addition of equal volume of 4% SDS to each 1 ml incubation mixture containing 2.0mg protein. Aliquot of 1.8 ml was withdrawn and passed through a Sephadex column, 40 x 3 cm. Pooled protein fractions were precipitated by addition of 2 volumes of cold acetone to wash out the detergent, mounted on planchets and counted.

An explanation for the poorer yield perhaps may be that the incorporated P^{32} was more unstable at a neutral pH (Fig. 2). Another possibility is that some step(s) in the reaction sequence (Eq. I) might not come to a complete standstill in

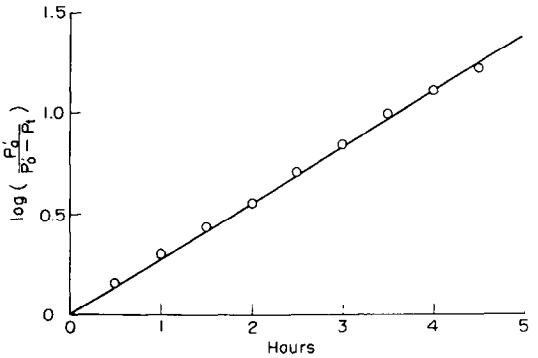

Fig. 1. Time course of P^{32} liberation from labeled protein

The ATPase preparation was labeled in the Na-system for 5 seconds. Protein concentration was 5.4 mg in 2 ml reaction mixture. The labeled protein was suspended in cold acetate buffer, pH 3.5, and held at 40°. Aliquots were sampled at times indicated, PCA added to 0.5N, and the supernate was counted. In the ordinate, p_t represents released P^{32} at time t; p_o' is the corrected value of the total bound P^{32} at the beginning (p_o). The correction was 4% ($p_o' = 0.96p_o$). The straight line was calculated according to the formula:

$$\log p_o'/(p_o' - p_t) = kt; \quad k = 4.63 \times 10^{-3}\text{min}^{-1}.$$

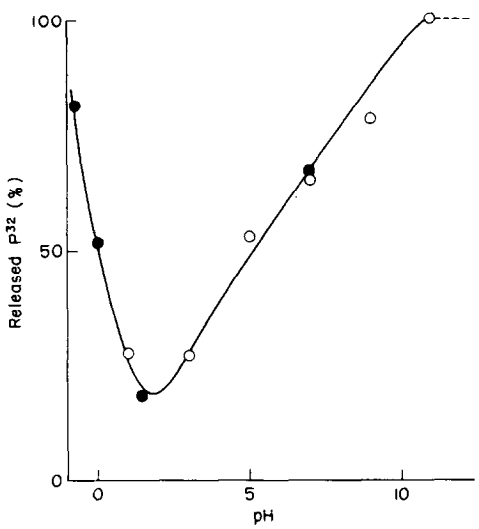

Fig. 2. Effect of pH upon the rate of liberation of P^{32} from the labeled ATPase preparation

Labeling conditions were as in Fig.1 except for the protein concentration. The open and solid circles represent different runs (open circles: 3.2mg protein in 1 ml; solid circles: 2.5 mg in 1 ml). The labeled protein was hydrolyzed for 30 min. at 40° in the following media: 6N HCl; 1N HCl; 0.05N HCl; 25 mM Na-citrate buffer, pH 3.0 and 5.0; 25mM Tris-maleate buffer, pH 7.0; 25mM Tris-HCl buffer, pH 9.0; and 0.1N K_2CO_3.

2% SDS. "That less than one turnover at an active site may give labeling" has already been pointed out (11).

b) Hydrolytic Properties of the Incorporated P^{32}

The bound P^{32} was converted to a PCA non-precipitable form when boiled for 10 min. in 1N mineral acids. It was even more labile in alkaline condition. In 0.1N NaOH, almost complete release occurred within a few minutes at room temperature. In both acidic and alkaline conditions the predominant radioactive product was Pi^{32}.

The time course of P^{32} liberation at 40°, pH 3.5, indicated that 96% of it was present as a single chemical species, as the hydrolysis followed the first-order reaction kinetics (Fig. 1).

The entire pH-lability profile (Fig. 2) showed an interesting pattern with a minimum at weakly acidic pH. This characteristic made it unlikely that the binding site was a serine or a histidine residue. Presence of the P^{32} as a thiophosphate compound seemed to be excluded as well, for butylthiophosphate is known to have a very different kind of pH-hydrolysis relationship (4).

c) Effect of Hydroxylamine upon P^{32} Release

The profile in Fig. 2 suggested an acyl phosphate linkage, for it resembles acetyl phosphate (7) in the increase of lability towards both extremes of pH scale. It may be, however, that structural factors exert profound effects upon the hydrolytic behavior of the bond.

For the purpose of more direct detection of the acyl ester, hydroxylaminolysis was tried (15). All the P^{32} was released as Pi after 10 min. incubation, when only several per cent liberation had occurred in the control groups (Table 2).

Thus, the presence of the incorporated P^{32} as an acyl ester-type compound in the phosphorylated intermediate of the Na^+-K^+-activated ATPase seems plausible. Further investigations on these and other facets of the labeled intermediary complex are now in progress.

Table 2. Accerelation of P^{32} liberation from phosphorylated ATPase preparation by hydroxylamine

	Additions	Released P^{32} after treatment		Ratio of Pi^{32} to released total radioactivity (%)
		Counts/3min.	Per cent to total incorporated P^{32}	
a.	None	365;379	6.1	-
b.	NaCl	371;396	6.3	-
c.	Hydroxylamine	5544;5312	89	92

Labeling conditions were as in Fig. 1. Hydroxylamine-HCl was neutralized and used as described for acetyl phosphate determination (15). In b group, to check the possible effect of ionic strength, NaCl was added to 1.3M, which was the same concentration expected to be introduced accompanying neutralized hydroxylamine solution.

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