K^+-STIMULATED INCORPORATION OF ^{32}P FROM NITROPHENYL PHOSPHATE INTO A $(Na^+ + K^+)$-ACTIVATED ATPase PREPARATION*

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Summary: To study an enzyme-phosphate intermediate in the K^+-phosphatase activity of an ATPase preparation, acidic pH's were chosen, where lessened discrimination between substrates indicated preferential slowing of dephosphorylation. At pH 5, KCl stimulated the incorporation of ^{32}P from nitrophenyl phosphate, and this increment in labeling was sensitive to ouabain, disappeared on brief incubations at pH 7.5, and was largely insensitive to hydroxylamine. NaCl also stimulated labeling, but best at neutral pH, and, like Na^+-stimulated labeling with ^{32}P-ATP, was sensitive to hydroxylamine. Insofar as the K^+-phosphatase reflects the terminal steps of the ATPase, the data suggest a K^+-activated transfer to the hydrolytic site after the initial Na^+-activated formation of an acyl phosphate.

Kinetic studies on the K^+-dependent phosphatase activity associated with a $(Na^+ + K^+)$-activated ATPase preparation indicated the existence of an enzyme-phosphate intermediate (1). Since this phosphatase activity appears to reflect the terminal K^+-dependent dephosphorylation steps of the $(Na^+ + K^+)$-activated ATPase (2, 3), it seemed of interest to look for such an intermediate and to consider its place in the overall reaction scheme of the ATPase.

Methods and Materials. The $(Na^+ + K^+)$-activated ATPase was prepared from rat brain (4). ATPase, AcPase, and NPPase activities were determined as previously described (3-5). Incorporation of ^{32}P from

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Abbreviations used: AcP, acetyl phosphate; AcPase, acetyl phosphatase; NPP, nitrophenyl phosphate; NPPase, nitrophenyl phosphatase.

(γ-^{32}P)-ATP and ^{32}P-NPP was measured following the procedure of Post et al. (6): after incubation at 30° the reaction was stopped by adding ice cold CCl_3COOH containing unlabeled substrate, and the mixture was centrifuged at 10,000 g for 5 min. The resulting pellet was washed four times with CCl_3COOH and finally dissolved in 1 N NaOH at 80°; aliquots were taken for measuring radioactivity and for protein determination (7). Values are corrected for ^{32}P incorporation in zero-time incubations. (γ-^{32}P)-ATP (23 Ci/mmole) was obtained from New England Nuclear, ^{32}P-NPP (3.5-5.3 mCi/mmole) from Amersham-Searle, and unlabeled substrates from Sigma; all were converted to the tris salts before use.

Results and Discussion. Since the K^+-activated phosphatase hydrolyzes different substrates at different rates, the rate-limiting step must be the formation of the presumed phosphate intermediate; hence, attempts to demonstrate such an intermediate would be favored by conditions whereby dephosphorylation is slowed related to phosphorylation. As the pH of the incubation medium was lowered from 8 to 5, hydrolytic activity fell, but also the relative rates of hydrolysis of the two phosphatase substrates became closer (Fig. 1): the ratio of NPPase to AcPase activity rose progressively from 0.13 (pH 9) to 0.68 (pH 5). (Na^++K^+)-activated ATPase activity also fell, nearing the rates of phosphatase activity as well.

In the absence of added monovalent cations the incorporation of ^{32}P from ^{32}P-NPP varied little with pH (Table 1) and averaged 0.1 nmole/mg protein at pH 7.8; omitting $MgCl_2$ from the medium reduced incorporation 70%. This labeling presumably represents extraneous activities contaminating this (3,4), as most, ATPase preparations; however, a fraction may reflect tightly-bound K^+ in the preparation (8) endogenously activating the K^+-phosphatase.

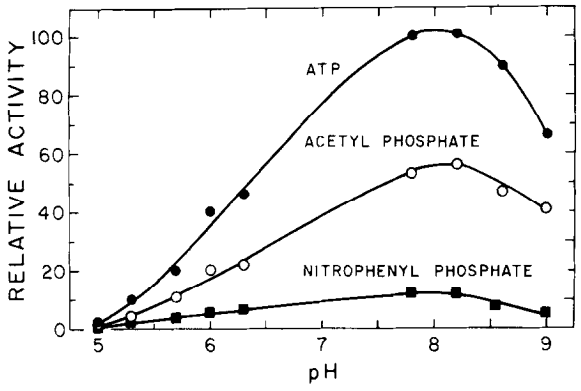

Fig. 1. Variation in activity with pH. Activities were measured during brief incubations at 30° in media containing 50 mM buffer at the pH indicated (tris-HCl above pH 7, tris-maleate below), 3 mM $MgCl_2$, 3 mM substrate as the tris salts, and either 90 mM NaCl plus 10 mM KCl with ATP or 10 mM KCl with AcP or NPP. Activity is presented in terms of μmoles of product formed (or substrate consumed) per min per mg protein, relative to that of ATP hydrolysis at pH 7.8 defined as 100. All values are corrected for activity in the absence of added monovalent cations.

Table 1. Incorporation of ^{32}P from nitrophenyl phosphate. Incorporation of ^{32}P was measured after 2-min incubations at 30° in media containing 50 mM buffer at the pH indicated (tris-HCl above 7, tris-maleate below), 1 mM $MgCl_2$, 1 mM ^{32}P-NPP, and the additions indicated. Control activity is presented in comparison with incorporation at pH 7.8, and the effects of additions are presented relative to the control activity at that pH, in all cases in terms of nmoles P_i incorporated per mg protein. Data are presented ± S.E.M. with the number of experiments, performed in duplicate, in parenthesis.

pH	Control activity	Addition	Concentration (mM)	Effect of addition	
5.0	1.3	none		(100)	
		KCl	20	180 ± 18	(7)
		NaCl	20	117 ± 6	(7)
5.7	1.1	none		(100)	
		KCl	20	95 ± 8	(6)
		NaCl	20	110 ± 6	(5)
7.8	1.0	none		(100)	
		KCl	20	78 ± 10	(5)
		NaCl	20	167 ± 9	(6)
		KCl + NaCl		113 ± 7	(5)
9.0	0.9	none			
		KCl	20	84 ± 8	(4)
		NaCl	20	150 ± 8	(6)

K^+-stimulated labeling was apparent at pH 5.0 (Table 1); the level of labeling was constant over the 1 to 5 minute period examined. However, this K^+-stimulated increment disappeared on raising the pH of the incubation mixture after an initial incubation at pH 5.0 (Table 2). These observations are consistent with a reaction scheme (5) in which K^+ activates the formation of a phosphorylated intermediate that is subsequently hydrolyzed: at an acidic pH, where dephosphorylation is slowed relative to phosphorylation, such an intermediate should be demonstrable, while at neutral pH rapid hydrolysis is favored.

Table 2. Loss of labeling at neutral pH. Experiments were performed at pH 5.0 as in Table 1 except that in specified cases at the end of the 2-min incubation additional buffer was added to affect the measured pH of the medium as indicated and the mixture was then incubated an additional 2 min (in these cases the volume of the incubation medium was increased 25%, and no corrections were made for the resulting dilution). Data are expressed relative to the incorporation of ^{32}P in the control medium incubated for 2 min.

Initial Conditions	Second incubation	pH	Relative incorporation of ^{32}P	
pH 5.0: control	no		(100)	
	yes	5.0	92 ± 7	(4)
	yes	7.5	82 ± 8	(4)
pH 5.0: plus 20 mM KCl	no		172 ± 14	(4)
	yes	5.0	158 ± 9	(4)
	yes	7.5	93 ± 9	(4)

In the presence of 0.1 mM ouabain, which inhibits this K^+-activated NPPase (9), adding KCl did not increase ^{32}P incorporation at pH 5.0 beyond control levels with ouabain alone.

In contrast to this reaction scheme for the K^+-activated phosphatase, kinetic studies (5, 10) suggest that a reaction pathway analogous to that for the $(Na^+ + K^+)$-activated ATPase is available to NPP in the presence of both

Na^+ and K^+. Moreover, a Na^+-stimulated phosphorylation was observed (Table 1) that may be identical to the initial step in the $(Na^+ + K^+)$-activated ATPase: formation of a glutamyl phosphate complex (11). The smaller increment in ^{32}P incorporation with NaCl reported previously (12) may have resulted from the presence of Na^+ (from the NPP) in the control medium.

Distinction between the two postulated phosphorylated forms, (i) the Na^+-activated formation of an acyl phosphate and (ii) the K^+-activated labeling, was approached in terms of sensitivity to hydroxylamine (Table 3). With both ATP and NPP the Na^+-activated increment in labeling was largely sensitive to hydroxylamine, whereas the K^+-stimulated increment was much

Table 3. Effect of hydroxylamine on ^{32}P incorporation from NPP and ATP. Incubations with ^{32}P-NPP were performed as in Table 1; incubations with ^{32}P-ATP were performed similarly but with 0.5 mM $MgCl_2$ and 0.1 mM ATP and for 1 min. After the washings with CCl_3COOH the pellet was resuspended in 1 ml of freshly-prepared 1 M NH_2OH, and incubated for 20 min at room temperature; CCl_3COOH was then added to a final concentration of 5% and the mixture was centrifuged. Radioactivity in the supernatant material and in the pellet was then measured. Data are presented in terms of (a) the total incorporation (NH_2OH-released ^{32}P + pellet ^{32}P) relative to the corresponding control, and (b) the percentage of total ^{32}P that was released by NH_2OH.

Substrate	pH	Addition	Relative incorporation	% liberated by NH_2OH	
^{32}P-NPP	5.0	none	100	28 ± 5	(5)
		KCl, 20 mM	176 ± 15	33 ± 6	(5)
	7.8	none	100	32 ± 5	(5)
		NaCl, 20 mM	162 ± 8	58 ± 6	(5)
^{32}P-ATP	5.0	none	100	24 ± 6	(4)
		KCl, 20 mM	108 ± 8	26 ± 6	(4)
	7.8	none	100	31 ± 5	(5)
		NaCl, 20 mM	522 ± 24	72 ± 6	(5)

less so. Attempts to distinguish different patterns of labeling in peptic digests of the phosphorylated protein were frustrated by the small quantities of radioactivity incorporated from the low specific activity ^{32}P-NPP.

For the K^+-activated phosphatase these experiments fit a model describing a K^+-activated phosphorylation that, at neutral pH, is rapidly hydrolyzed; in the presence of both Na^+ and K^+ a reaction pathway analogous to that of the ATPase becomes available, with an initial Na^+-activated formation of an acyl phosphate followed by a K^+-activated transfer to the hydrolytic site (5). Direct entry to the hydrolytic site would be available to the phosphatase substrates in a K^+-activated step; the failure of ATP to undergo significant K^+-activated hydrolysis (or labeling) would reflect difficulties in direct access of the entire ATP molecule to the terminal hydrolytic site.

References

1. Robinson, J. D., Biochim. Biophys. Acta, 212, 509 (1970).
2. Askari, A. and Koyal, D., Biochem. Biophys. Res. Commun., 32, 227 (1968).
3. Robinson, J. D., Biochemistry, 8, 3348 (1969).
4. Robinson, J. D., Biochemistry, 6, 3250 (1967).
5. Robinson, J. D., Arch. Biochem. Biophys., 139, 164 (1970).
6. Post, R. L., Sen, A. K., and Rosenthal, A. S., J. Biol. Chem., 240, 1437 (1965).
7. Lowry, H. O., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
8. Goldfarb, P. S. G. and Rodnight, R., Biochemical J., 120, 15 (1970).
9. Robinson, J. D., Mol. Pharmacol., 5, 585 (1969).
10. Robinson, J. D., Arch. Biochem. Biophys., 139, 17 (1970).
11. Kahlenberg, A., Galsworthy, P. R., and Hokin, L. E., Science, 157, 434 (1967).
12. Inturrisi, C. E. and Titus, E., Mol. Pharmacol., 6, 99 (1970).