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ON THE POSSIBLE ROLE OF THE PHOSPHORYLATED INTERMEDIATE IN THE REACTION MECHANISM OF (Na^+-K^+)-ATPase

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SUMMARY

1. Phosphorylation and dephosphorylation of (Na^+-K^+)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) by [γ-^{32}P]ATP was examined using highly specific NaI-treated preparations from pig-brain microsomes.

2. The phosphorylated enzyme consisted of an essentially single chemical species. The incorporated phosphate was relatively stable in an acidic medium (pH 1–2), and was easily released from the enzyme by treatment with hydroxylamine at a neutral pH.

3. The chemical nature of the phosphate bond was similar under the following alternative conditions: (a) whether the labeling was stopped by $HClO_4$ or dodecyl sulfate; (b) whether K^+ was present or not in addition to Na^+; or (c) whether the labeling was performed at 37° or 0°.

4. Longer incubation of the enzyme preparation with [γ-^{32}P]ATP resulted in another form of incorporation which was hydroxylamine-resistant.

5. The high specificity of the ATP-hydrolyzing reaction of (Na^+-K^+)-ATPase to Na^+ and K^+ was partially lost at 0°, while the specific response of the phosphorylated enzyme to Na^+ and K^+ was still maintained.

6. Based on these results, possible relationships of the phosphorylated (Na^+-K^+)-ATPase to the ATP-hydrolyzing reaction of the enzyme are discussed.

INTRODUCTION

(Na^+-K^+)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparations from various sources are labeled by the terminal phosphate of [γ-^{32}P]ATP (*cf.* refs. 1–8). Several authors have assumed the phosphorylated enzyme to be an intermediate of the (Na^+-K^+)-ATPase reaction, whereas others have considered it could not be[9,10].

We have obtained a highly specific (Na^+-K^+)-ATPase from brain microsomes by treatment with a high concentration of NaI solution[11], and using this preparation, have found that the phosphate is possibly bound to the enzyme through an acyl-phosphate linkage[5]. Deoxycholate-treated (Na^+-K^+)-ATPase preparations have also been reported to form a similar enzyme–phosphate complex[12,13]. The fact that the bound phosphate is easily transferred to methanol to form methylphosphate in acidic

condition[13,14] like ATP (*cf.* ref. 15) also suggests the high-energy nature of the phosphate bond.

Detailed chemical and kinetic studies of the complex are still required before it can be concluded whether or not the enzyme–phosphate complex is an intermediate of, or a complex directly related to, the ATP-hydrolyzing reaction. In the present report some of our experiments dealing with properties of the ^{32}P-labeled NaI-treated enzyme formed under various conditions and its relation to the ATP-hydrolyzing reaction will be described and the plausibility of alternative interpretations will be discussed.

MATERIALS AND METHODS

Enzyme preparation

Brain microsomal fraction was the enzyme source. Pig brain was obtained from the slaughter-house and stored at —20° before use. The heavy microsomal fraction was obtained from the cerebrum and treated with a high concentration of NaI solution as described previously[11]. The enzyme, washed free from traces of remaining NaI by repeated centrifugation, was fairly stable under storage at —20°. Recovery was 80–120 mg protein in the final preparation from 100 g of cerebral tissue. The stocked enzyme suspension was thawed immediately before use and was lightly homogenized with a Teflon homogenizer.

Terminally labeled [^{32}P]ATP

[^{32}P]ATP was prepared according to GLYNN AND CHAPPELL[16] with slight modifications. The eluate from Dowex 1 column containing the terminally labeled free [^{32}P]ATP was neutralized with 2 M Tris solution and stored at —20°.

Assay conditions and determination of specific activity of enzyme

ATPase activity was assayed, unless otherwise specified, in a standard assay medium[11] containing 3 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA–Tris, 20 mM Tris–HCl buffer at pH 7.8, and KCl and NaCl as indicated, for 30 min at 37°. The reaction was linear for at least 60 min under these conditions.

Released P_i was determined according to a slight modification of the method of FISKE AND SUBBAROW after deproteinization with 3 % $HClO_4$. Protein was determined as described by LOWRY *et al.*[17].

$^{32}P_i$ was determined by extraction into isobutanol–benzene mixture (1:1, v/v) as described by MARTIN AND DOTY[18].

Labeling of the enzyme

The reaction was initiated by the addition of [^{32}P]ATP to the mixture in a flat-bottomed test tube held in a water-bath and stirred by a magnetic bar. ATP and other solutions were blown into the test tube at successive intervals through small pipettes connected to mechanically driven pistons. Solutions in the pipettes, except $HClO_4$, were held at the same temperature as the water-bath by means of circulating-water mantles. Reaction was performed in a final volume of 1 ml, and stopped by addition of 3 vol. of cold 4 % $HClO_4$ or an equal vol. of 4 % sodium dodecylsulfate.

Radioactivity determination of labeled enzyme

The enzyme suspension in $HClO_4$ was centrifuged at 10000 × *g* for 7 min in a refrigerated centrifuge. The precipitate was washed three times with 3 % $HClO_4$ containing 10 mM P_i and 0.1 mM ATP as carriers. The final pellet was taken up and counted in a low-background gas-flow counter.

Treatment by hydroxylamine and hydrolysis of the labeled enzyme were performed as described previously[5].

Labeled enzyme obtained by addition of sodium dodecylsulfate was separated from the remaining [^{32}P]ATP and $^{32}P_i$ through a Sephadex G-25 column[5].

RESULTS

Comparison of the phosphorylated enzyme preparations obtained under various conditions

Recovery of the phosphorylated enzyme obtained by termination of the reaction by $HClO_4$ or sodium dodecylsulfate. Increase in the amount of the phosphorylated enzyme by addition of Na^+ and its decrease by K^+ have repeatedly been observed when the labeling was stopped either by $HClO_4$ (*cf.* refs. 1–8) or sodium dodecylsulfate (*cf.* ref. 6). The labeling reached a plateau in a few seconds. A systematic comparison between termination of the labeling by $HClO_4$ and sodium dodecylsulfate after 5 sec incubation is shown in Table I. The effects of Na^+ and K^+ are parallel between the terminations by $HClO_4$ and sodium dodecylsulfate. When K^+ was added simultaneously with sodium dodecylsulfate to stop the labeling reaction, the recovery of the phosphorylated enzyme was diminished (Table II). This result indicates that, when sodium dodecylsulfate was added to the system, there existed a short delay before the reaction came to a complete standstill.

Chemical identity between the phosphorylated enzyme obtained by $HClO_4$ and sodium dodecylsulfate. The phosphorylated enzyme obtained by sodium dodecylsulfate had the following properties. (1) The pH-lability profile of the phosphate–enzyme bond showed a characteristic pattern having the relatively most stable region near pH 1–2

TABLE I

RECOVERY OF THE PHOSPHORYLATED ENZYME AFTER ADDITION OF EITHER $HClO_4$ OR SODIUM DODECYLSULFATE

Enzyme (0.7 mg protein) was incubated with 13 μM [^{32}P]ATP for 5 sec at 0°. Labeling was stopped by addition of 3 vol. of 4 % $HClO_4$ or an equal vol. of 4 % sodium dodecylsulfate. Procedures for determination of the phosphorylated enzyme were as described under MATERIALS AND METHODS. Values in parentheses are per cent amounts of the phosphorylated enzyme formed in the presence of Na^+ alone as 100. Zero-time values (60 mμmoles P per g protein for $HClO_4$ and 3 mμmoles P per g protein for sodium dodecylsulfate) were subtracted from all values in the table.

Cations (mM)		*Recovery of phosphorylated enzyme (mμmoles P per g protein)*	
Na^+	*K^+*	*$HClO_4$*	*Sodium dodecylsulfate*
0	0	56 (15)	103 (32)
0	14	54 (13)	23 (7)
140	14	123 (31)	76 (24)
140	0	392 (100)	338 (100)

TABLE II

EFFECT OF SIMULTANEOUS ADDITION OF KCl WITH SODIUM DODECYLSULFATE ON RECOVERY OF THE PHOSPHORYLATED ENZYME

Enzyme (2.3 mg protein) was incubated with 33 μM [^{32}P]ATP for 5 sec at 0°. Labeling was stopped by addition of an equal volume of 4 % sodium dodecylsulfate containing or not containing 1.4 mM KCl. The sodium dodecylsulfate solution was kept at 10° before addition since precipitates of potassium dodecylsulfate appeared at lower temperatures. Zero-time value (3 mμmoles P per g protein) was subtracted from the values in the table.

KCl in sodium dodecylsulfate (mM)	*Phosphorylated enzyme (mμmoles P per g protein)*
0	237
1.4	107

TABLE III

RELEASE OF PHOSPHATE FROM THE PHOSPHORYLATED ENZYME OBTAINED BY ADDITION OF SODIUM DODECYLSULFATE

The phosphorylated enzyme in sodium dodecylsulfate was precipitated with carrier (Na^+-K^+)-ATPase protein by addition of acetone at −20°. The precipitated enzyme was washed with 3 % $HClO_4$, then with water, by centrifugation, and treated with hydroxylamine. Hydroxylamine hydrochloride (1.4 g) was dissolved in 5 ml of 100 mM Tris–maleate buffer (pH 6.5), neutralized with 4 M NaOH, adjusted to 10 ml with water, and 0.5 ml of this solution was added to 1 ml of the enzyme suspension. After 10 min at 20°, 1 ml of 4 % $HClO_4$ was added, and the supernatant after centrifugation was counted. The control group was treated similarly with 50 mM Tris–maleate buffer (pH 6.5).

Addition	*Release of phosphate from the phosphorylated enzyme*		
	Released phosphate (mμmoles per g protein)	*% of total incorporation*	*Ratio of $^{32}P_i$ to released total counts*
Buffer	22	8	—
Hydroxylamine	251	100	88

TABLE IV

HYDROXYLAMINOLYSIS OF THE PHOSPHORYLATED ENZYME FORMED IN THE PRESENCE OR ABSENCE OF K^+

Enzyme (1.4 mg protein) was incubated with 11 μM [^{32}P]ATP in the presence of 140 mM Na^+ or 140 mM Na^+ + 14 mM K^+ for 5 sec at 0°. The phosphorylated enzyme was recovered by addition of $HClO_4$ and treated with hydroxylamine or control buffer. The conditions of the treatment were as in Table III. The results are expressed in % of the total incorporated counts in the enzyme.

Cations	*Recovery of phosphorylated enzyme (mμmoles P per g protein)*	*Released ^{32}P (%) from phosphorylated enzyme incubated with:*	
		Buffer	*Hydroxylamine*
Na^+	198	9.8	99.5
$Na^+ + K^+$	43	7.9	95.0

(Fig. 1). (2) The enzyme consisted of an essentially single chemical species as indicated by the first-order hydrolysis kinetics at pH 3.5 at 40° (Fig. 2). The half-hydrolysis time under these conditions was about 41 min. (3) It was liable to hydroxylaminolysis at pH 6.5 at 20°, and released its label as $^{32}P_i$ (Table III).

These properties are similar to those of the phosphorylated enzyme obtained by $HClO_4$ as reported earlier[5].

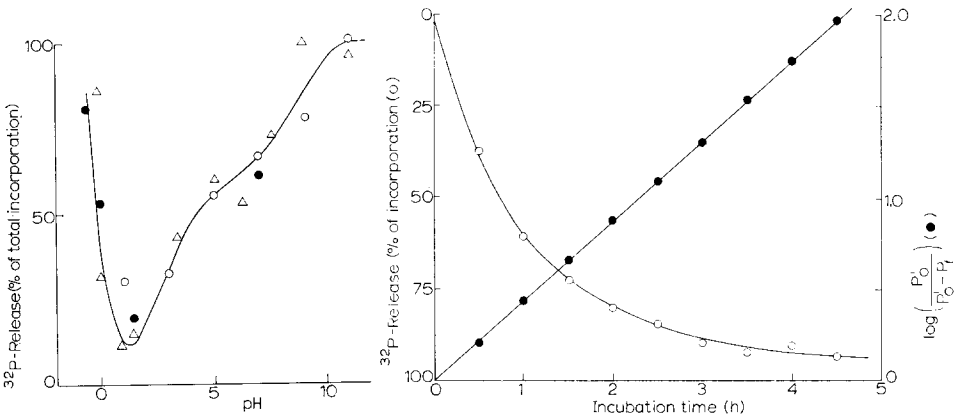

Fig. 1. Effect of pH upon the rate of phosphate liberation from phosphorylated enzyme. The enzyme (2.5–3.4 mg protein per ml) was labeled in the presence of Na^+ for 5 sec at 0°. The labeling was stopped by addition of $HClO_4$ or sodium dodecylsulfate, and separated as described under MATERIALS AND METHODS. Each aliquot of the labeled enzyme was incubated at the indicated pH for 30 min at 40° (ref. 5). At the end of incubation, $HClO_4$ was added to a final concentration of 0.5 M, and the ^{32}P released was measured in the supernatant after centrifugation. Results are expressed in % release of the total incorporation into the enzyme. Open and filled circles: two different lots of the phosphorylated enzyme recovered from $HClO_4$; triangles: phosphorylated enzyme recovered from sodium dodecylsulfate.

Fig. 2. Time course of ^{32}P liberation from the phosphorylated enzyme. Labeling of the enzyme (3.4 mg/ml) was stopped by sodium dodecylsulfate. The phosphorylated enzyme was suspended in acetate buffer (pH 3.5) and held at 40°. Aliquots were sampled out at times indicated, and the ^{32}P released was counted as described for Fig. 1. Filled circles show an apparent first-order kinetics calculated from the release curve based on the equation $\log p'_0/(p'_0 - p_t) = kt$; $k = 3.64 \cdot 10^{-3}$ min^{-1}, where p'_0 is the corrected value of the total bound ^{32}P at zero time (p_0). The correction was 4% ($p'_0 = 0.96p_0$). p_t represents ^{32}P released from the labeled enzyme at time t.

Comparison between phosphorylated enzyme obtained in the presence or absence of Na^+ and K^+. When K^+ was added to the system containing Na^+, the level of the phosphorylated enzyme was markedly diminished (Table I). However, the chemical property of the phosphate–enzyme bond seemed to remain the same irrespective of the presence or absence of K^+ as suggested by the essentially identical liability to hydroxylaminolysis of the incorporated phosphate (Table IV).

On the other hand, when both Na^+ and K^+ were omitted from the system, slow but gradually increasing labeling was observed at 37° (Fig. 3B) as well as at 0° (data not shown). A large part of the labeled enzyme formed in the absence of any alkali cations was hydroxylamine-insensitive (Table V) in contrast to that formed in the presence of Na^+ and/or K^+. The apparent steady-state rate of formation of this hydroxylamine-insensitive labeling was 7 mμmoles per g protein per sec as calculated

from Fig. 3B. The rate of (Na^+-K^+)-stimulated ATP-hydrolysis calculated from Fig. 3A was 7.3 μmoles per g protein per sec. Thus the former was far slower than the latter.

ATP-hydrolysis and the phosphorylated enzyme at low temperatures

Modification of response of (Na^+-K^+)-ATPase to Na^+ and K^+ at low temperatures. The high specificity of (Na^+-K^+)-ATPase to the simultaneous addition of Na^+ and K^+ was partially lost at low temperatures[7–9,19] as seen in Figs. 4 and 5. Addition of Na^+ alone accelerated the hydrolysis considerably (Fig. 4A), while the supplementation of K^+ to Na^+ (Fig. 5A) further accelerated it only to a limited extent (15–40 % in five instances).

In Figs. 4 and 5, ATP concentration was fairly low for technical reasons. However, the modification in cation specificity was not brought about merely by the low concentration of ATP, since the apparent loss of strict specificity to Na^+ and K^+ at 0° was also observed at an ATP concentration of 3 mM (Table VI).

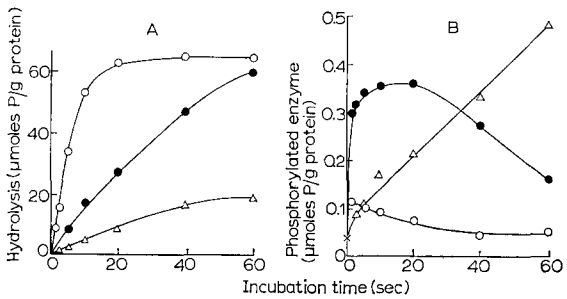

Fig. 3. ATP-hydrolysis and formation of the phosphorylated enzyme in the presence or absence of Na^+ and/or K^+ at 37°. The reaction was performed in the presence or absence of Na^+ and/or K^+ using 1.5 mg of the enzyme protein and 0.1 mM [^{32}P]ATP at 37°. The reaction was stopped by $HClO_4$. Hydrolysis of [^{32}P]ATP was determined by using an aliquot of each supernatant after centrifugation (Fig. 3A). The precipitate was washed by repeated centrifugation, and the radioactivity counted (Fig. 3B). Open circles, with 140 mM Na^+ and 14 mM K^+; filled circles, with 140 mM Na^+; triangles, without Na^+ or K^+; cross, zero-time value of the phosphorylated protein.

TABLE V

COMPARISON OF HYDROXYLAMINE-SENSITIVITY AMONG THE PHOSPHORYLATED ENZYMES FORMED UNDER DIFFERENT LABELING CONDITIONS

Enzyme (0.9 mg protein) was incubated with 0.1 mM [^{32}P]ATP for the indicated period at 0° or 37° in the presence of 140 mM Na^+ or 14 mM K^+ as indicated. The reaction was stopped by $HClO_4$. The phosphorylated enzyme was incubated with hydroxylamine as described in the legend to Table III.

Temperature	*Cations*	*Incubation time (sec)*	*Phosphorylated enzyme (mμmoles P per g protein)*	*Released ^{32}P (% of total incorporation)*
0°	—	300	50	21
	Na^+	10	300	76
	$Na^+ + K^+$	5	110	85
37°	—	60	240	35
	Na^+	5	210	82
	$Na^+ + K^+$	1	90	88

Rate of phosphorylation and dephosphorylation of the enzyme at 0°. The enzyme was rapidly phosphorylated and dephosphorylated at 0° as well as at 37°. Fig. 6A shows the phosphorylation and ATP-hydrolysis in a short-time reaction at 0° in the complete system (Na^+ and K^+ present). The enzyme was phosphorylated maximally before 0.5 sec. The minimal estimate of the rate of the phosphorylation (the upper broken line of Fig. 6A) is 0.13 μmole per g protein per sec under these conditions.

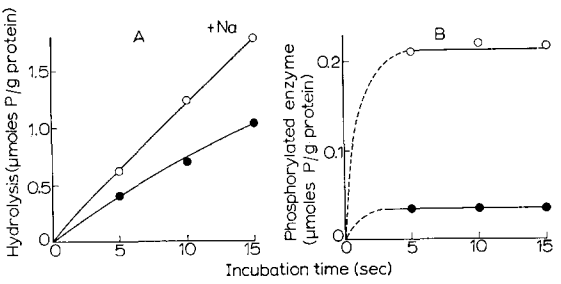

Fig. 4. ATP-hydrolysis and formation of the phosphorylated enzyme in the presence or absence of Na^+ at 0°. The procedure was similar to that for Fig. 3 except for the following conditions. Enzyme, 0.74 mg/ml; ATP, 13.5 μM; incubation temperature, 0°. A, ATP-hydrolysis; B, phosphorylated enzyme. Open circles, with 140 mM Na^+; filled circles, without Na^+.

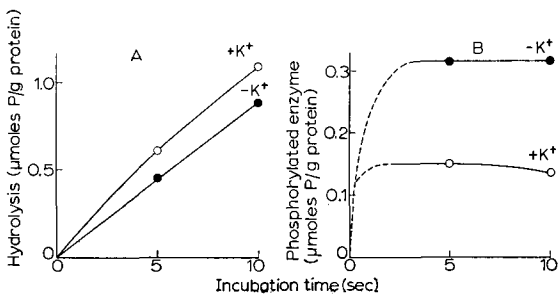

Fig. 5. ATP-hydrolysis and formation of the phosphorylated enzyme in the presence or absence of K^+ at 0°. The procedure was similar to that for Fig. 4 except for the enzyme concentration (0.94 mg/ml). A, ATP-hydrolysis; B, phosphorylated enzyme. Open circles, with 140 mM Na^+ and 14 mM K^+; filled circles, with only 140 mM Na^+.

Fig. 6B shows that the phosphorylated enzyme formed in the absence of K^+ was dephosphorylated rapidly when K^+ was added to the system. The half-dephosphorylation time was less than 0.5 sec. The apparent rate constant k, as defined by the formula

$$-\frac{dp}{dt} = k \cdot p$$

(p = amount of the phosphorylated enzyme in μmoles/g protein) is calculated as 1.9 sec^{-1} from the drop in the amount of the phosphorylated enzyme during the first 0.5 sec after K^+ was added (broken line of Fig. 6B). If we assume the steady-state level of the phosphorylated enzyme as 45 mμmoles per g protein (estimated from the plateau values in Figs. 6A and B), then the rate of release of $^{32}P_i$ from the phosphorylated enzyme under these conditions is calculated as 85 mμmoles per g protein per sec. This value is about a half the total rate of ATP-hydrolysis calculated from the hydrolysis curve in Fig. 6A (0.15 μmole per g protein per sec).

An experiment similar to that represented in Fig. 6B was performed at 25° to ascertain the effect of ouabain (data not shown). The preformed phosphorylated enzyme did not decrease but maintained the zero-time level 2 sec after the addition of K^+ when 0.1 mM ouabain was included in the incubating mixture.

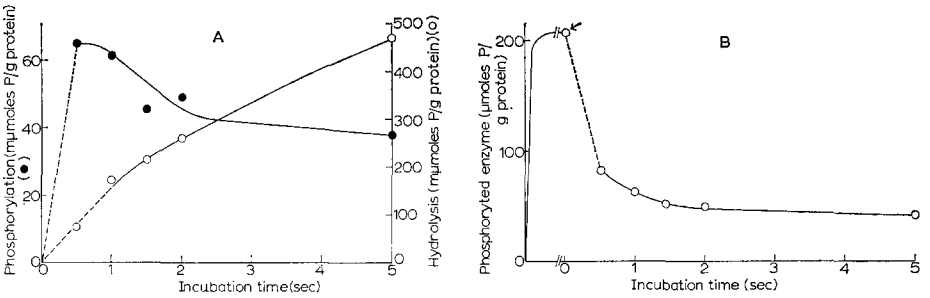

Fig. 6. Phosphorylation and dephosphorylation of (Na^+-K^+)-ATPase at 0°. The procedure of the reaction was as described under MATERIALS AND METHODS. ATP-hydrolysis and the phosphorylated enzyme were measured as described for Fig. 3. In Fig. 6A, 140 mM Na^+ and 14 mM K^+ were present from the beginning of the reaction. In Fig. 6B, the enzyme was phosphorylated at first in the presence of Na^+ alone for 5 sec, then K^+ was added at the arrow mark.

TABLE VI

TEMPERATURE DEPENDENCY OF ION SPECIFICITY OF (Na^+-K^+)-ATPase

ATPase activity was measured in the standard assay medium using 600 μg of the enzyme protein per tube. The incubation time was 30 sec at 37° or 130 min at 0°. Values in parentheses are relative specific activities expressed in % of the complete system.

Cations	*Specific activity (μmoles P per mg protein per h)*	
	37°	*0°*
—	3.3 (3.4)	0.16 (64)
Na^+	7.0 (7.2)	0.22 (88)
K^+	3.7 (3.8)	0.17 (68)
$Na^+ + K^+$	97.5 (100.0)	0.25 (100)

DISCUSSION

Many authors have considered that phosphorylation of (Na^+-K^+)-ATPase represents formation of an intermediate of the (Na^+-K^+)-stimulated ATP-hydrolyzing reaction. Results consistent with reaction mechanisms identical or similar to the following scheme have been reported[1–8]:

$$E + ATP \xrightarrow{Na^+} EP \xrightarrow{K^+} E + P_i \qquad (I)$$

Recently several difficulties have been pointed out by several other authors[9,10,19] that make this simplified scheme unacceptable. KANAZAWA, SAITO AND TONOMURA[19] have analyzed the partial loss of the cation specificity of (Na^+-K^+)-ATPase at low temperatures and proposed a modified reaction mechanism. Their suggested mechanism was essentially the same as that for myosin B recently proposed by the same

group of investigators[20–22], namely (Na^+-K^+)-ATPase hydrolyzes ATP through two different types of reaction, that is, through simple hydrolysis and hydrolysis *via* a reactive enzyme–phosphate complex. The latter type of reaction is the only pathway when the reaction proceeds at 37°, while the former becomes more dominant at lower temperatures. The mechanism may be represented, in a simplified form, as follows:

$$E + ATP \begin{cases} \xrightarrow{Na^+} E + P_I & (a) \\ \xrightarrow{Na^+} EP \xrightarrow{K^+} E + P_I & (b) \end{cases} \qquad (II)$$

Many of our results at 0° reported in this paper were consistent with this revised scheme provided that reaction (b) did not stop completely but both (a) and (b) operated at 0°. The proviso is based on the observations that: (i) the chemical properties of the enzyme–phosphate complex seemed to be the same whether they were formed at 37 or 0°; (ii) the addition of K^+ accelerated the hydrolysis of ATP at 0°, although the apparent degree of the acceleration was much less than at 37°; and (iii) the addition of K^+ rapidly decomposed the preformed enzyme–phosphate complex at 0°.

Regarding the second point just cited, KANAZAWA, SAITO AND TONOMURA[19] reported that K^+ did not accelerate the hydrolysis rate at all at 0°, an observation at variance with our present result (Fig. 4A). However, it was our experience that the degree of acceleration by K^+ at 0° was somewhat variable from preparation to preparation. Other investigators[8] have also pointed out the variability.

Another criticism against a simplified reaction mechanism came from the work of SCHONER, KRAMER AND SEUBERT[10]. They have shown that, when one of several non-labeled di- or trinucleotides was added to the system, the level of the ^{32}P-labeled enzyme diminished, while the rate of ATP-hydrolysis was little affected. They compared the nucleotide effect on the phosphorylated enzyme and that on the hydrolysis in different conditions (K^+ was absent and [^{32}P]ATP concentration was 0.1 mM for phosphorylation; both Na^+ and K^+ were present and the ATP concentration was 3 mM for hydrolysis). Cationic conditions and ATP concentration should be the same in this kind of comparison, since they may induce some changes, presumably conformational, in the enzyme, as suggested from inhibition experiments of (Na^+-K^+)-ATPase by SH inhibitors[23] or DFP (*cf.* refs. 24, 25), or from the study on *p*-nitrophenyl phosphatase activity of (Na^+-K^+)-ATPase preparations[25,28].

In this connection, we have compared the phosphorylation and the ATP-hydrolysis under identical conditions. However, some kinetic non-parallelisms were still observed (to be published). Reaction mechanism II, as well as I, did not easily explain these results. Some further modifications may be required. For instance, two intermediates EP_I and EP_{II}, both of which become hydroxylamine-sensitive and thus indistinguishable when the protein is denatured, may need to be assumed instead of only one EP in the reaction IIb.

However, there are many lines of evidence in favor of the interpretation that the hydroxylamine-sensitive phosphorylated enzyme is an intermediate of, or has a direct relationship to, the (Na^+-K^+)-activated ATP-hydrolysis. Some of them are as follows. (a) Na^+ specifically increases, and K^+ specifically decreases, the level of the phosphorylated enzyme (*cf.* Table I; Figs. 4 and 5; also refs. 1–8). (b) Response of the phosphorylated enzyme to addition of Na^+ or K^+ is rapid enough to suggest

its involvement in the reaction (*cf.* Figs. 6A and B). (c) Ouabain inhibits K^+-dependent dephosphorylation at higher temperatures (*cf.* RESULTS; also ref. 6). (d) The phosphate is attached presumably to an acyl residue in the enzyme[5,12,13] on one hand; the enzyme attacks some acylphosphates and the hydrolysis is K^+-dependent[26–28] on the other. (e) The maximal level of phosphorylation and the rate of maximal hydrolysis are parallel in different batches of similarly treated enzyme preparations[4]. These observations make it rather implausible that there are no direct relationships between the phosphorylated enzyme and the (Na^+-K^+)-activated ATP-hydrolysis. However, some complications may be involved, as was suggested by several studies[9,10], and elucidation of the exact role of the phosphorylated enzyme remains to be investigated.

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