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# Formation of ADP-Sensitive Phosphorylated Intermediate in the Electric Eel Na,K-ATPase Preparation

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SUMMARY

The ADP-sensitive and $K^+$-sensitive phosphorylated forms of Na,K-ATPase ($E_1P$ and $E_2P$, respectively) are believed to be the main phosphorylated intermediates of Na,K-ATPase. In the presence of 100 mM $Na^+$, $E_2P$ is the major component of the phosphorylated form in all native Na,K-ATPase preparations known, including the microsomes of shark rectal glands. However, the Na,K-ATPase-rich membrane fragments of the electric eel formed a different type of phosphoenzyme, of which 80% was dephosphorylated by both 2 mM $K^+$ and 1 mM ADP within 1 sec at 4° under the same conditions. In the presence of oligomycin (50 μg/ml), only the percentage of $K^+$-sensitive phosphoenzyme was reduced in the eel enzyme, but no such effects were observed in the shark enzyme. However, the eel enzyme produced $E_2P$-rich phosphoenzyme in the presence of 10 mM $Na^+$, as did the shark enzyme in the presence of 100 mM $Na^+$, and the shark enzyme formed the $E_1P$-rich phosphoenzyme in the presence of 500–700 mM $Na^+$, as did the eel enzyme in the presence of 100 mM $Na^+$. These results suggest that the eel enzyme has a much higher affinity for $Na^+$ on the $Na^+$ site controlling the $E_1P$-$E_2P$ equilibrium than does the shark enzyme, but we have not been able to explain this difference. Since the phosphorylated forms of both enzymes became more sensitive to both ADP and $K^+$ at 25°, both the interconversion of $E_1P$ to $E_2P$ and the reverse reaction seem to be accelerated by an increase in temperature. Oligomycin inhibited this conversion of $E_1P$ to $E_2P$ at 25° as at 4°, but did not inhibit the reverse reaction. Therefore, we conclude that $E_1P$ was the predominant phosphorylated form of the eel enzyme under the above conditions, and that $E_1P$ was converted to $E_2P$ and then dephosphorylated by $K^+$.

## INTRODUCTION

Na,K-ATPase (EC 3.6.1.3) has been recognized as the ($Na^+ + K^+$)-pump by the demonstration of active transport in reconstituted Na,K-ATPase liposomes (1). The cleavage of ATP proceeds through phosphorylated intermediates—$E_1P$[1] and $E_2P$. The dephosphorylation of $E_1P$ or $E_2P$ is usually thought to be accelerated by ADP or $K^+$, respectively. It is generally believed that $E_2P$ is usually the main component of the phosphorylated enzyme, whereas $E_1P$ is the main component only in the presence of very high concentrations of $Na^+$ or in an enzyme partially inhibited by *N*-ethylmaleimide (2).

Among the many Na,K-ATPase preparations from various sources, the enzyme from the electric eel electric organ has exhibited several unique characteristics. For example, the association rate between the eel enzyme and ouabain is much less than that of the other enzyme preparations (3). We report here some other characteristics of the forms phosphorylated from ATP of the eel enzyme. Since eel Na,K-ATPase is extremely detergent-sensitive, we used Na,K-ATPase-rich membrane fragments produced without detergents (4) and compared these with microsomes of the shark rectal gland, which, when prepared without detergents, contain a fairly active form of Na,K-ATPase (5).

## MATERIALS AND METHODS

*Preparation of the enzyme.* The eel enzyme used was prepared from the Na,K-ATPase-rich membrane fragments obtained from the electric organ of the electric eel. This preparation was obtained as the higher active fraction of density gradient centrifugation (density 1.11–1.14). It was prepared without any detergent, and the specific activity of the preparation obtained was approximately 11 ± 2 μmoles of ADP per milligram per minute at 30° (4). The shark microsomes, which were the generous gift of Dr. L. E. Hokin (University of Wisconsin), were prepared with his methods (5) and had a specific activity of 1.5 μmoles of ADP per milligram per minute at 30°.

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[1] The abbreviations used are: $E_1P$, ADP-sensitive phosphorylated form of Na,K-ATPase; $E_2P$, $K^+$-sensitive phosphorylated form of Na,K-ATPase; CDTA, 1, 2-cyclohexylene dinitrilotetraacetic acid; EP, phosphorylated form of Na,K-ATPase.

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[γ-$^{32}$P]ATP was prepared by using the method of Glynn and Chappell (6) with the modifications of Post and Sen (7). ADP was obtained from P-L Biochemicals (Milwaukee, Wisc.) and Sigma Chemical Company (St. Louis, Mo.).[2] Oligomycin (according to the manufacturer, a mixture of 60% oligomycin A, 20% oligomycin B, and 15% oligomycin C) was purchased from Sigma Chemical Company. CDTA was purified by precipitating its alkaline solution with HCl.

*Phosphorylation of the enzyme.* The suspension (0.9 ml) in the reaction tube, containing the enzyme preparation (about 100 μg of eel enzyme or 200 μg of shark enzyme), NaCl as indicated, 3 μmoles of $MgCl_2$, and 30 μmoles of Tris-HCl (pH 7.6), was mixed with 0.1 ml of [γ-$^{32}$P]ATP solution (about 20 μM). After a 2-sec incubation at 4° or 25°, the phosphorylation was terminated by the addition of 0.5 ml of 150 mM CDTA solution (pH 7.6) with the same $Na^+$ concentration as in the original mixture with or without oligomycin (150 μg/ml). To accelerate the dephosphorylation, 0.1 ml of 16 mM ADP or 32 mM KCl was added either at the same time or a half-second later. The reaction was then terminated by the addition of 0.2 ml of 85% trichloroacetic acid solution containing 0.1 mM unlabeled ATP and 1 mM $P_i$ at the times indicated. To obtain an accurate reaction time, the semi-rapid mixing apparatus originally described by Kanazawa *et al.* (8) was utilized, the reaction temperature was maintained by circulating water at a constant temperature, and the reaction mixture was vigorously stirred.

After the reaction mixture was placed in ice for 30–40 min, 1 ml of the mixture was placed on a Millipore filter (0.45 μm pore size), washed with about 70 ml of ice-cold 5% trichloroacetic acid containing 1 mM $P_i$ and 0.1 mM unlabeled ATP, and washed again with 10 ml of ice-cold water (9). The washed precipitate, along with the filter, was solubilized by acetone in a counting vial, and its radioactivity was measured by the Cerenkov method.

For the phosphoprotein data shown here, except those in Fig. 8, each value represents an average of results of quadruplicate experiments. The amounts of phosphoprotein formed were determined by subtracting from the original amount the nonspecific phosphoprotein determined by measuring the amount of phosphoprotein present when the CDTA solution was added before the [γ-$^{32}$P]ATP solution. The error in the phosphoprotein assay was estimated to be less than 5%.

For convenience, the $E_1P$ and $E_2P$ percentages were calculated as the ratio of the difference between the phosphoenzyme values after a 1-sec dephosphorylation with 1 mM ADP or 2 mM $K^+$ in the absence of $K^+$ and ADP (cf. Figs. 3A and B, and 4).

## RESULTS

*$E_1P$ and $E_2P$ percentages of eel and shark enzymes.* As shown in Fig. 1, the eel enzyme phosphoprotein, formed in the presence of 10 or 1000 mM $Na^+$, was dephosphorylated biphasically in the presence of $K^+$ or ADP. The original content of $E_1P$ and $E_2P$ at zero-time

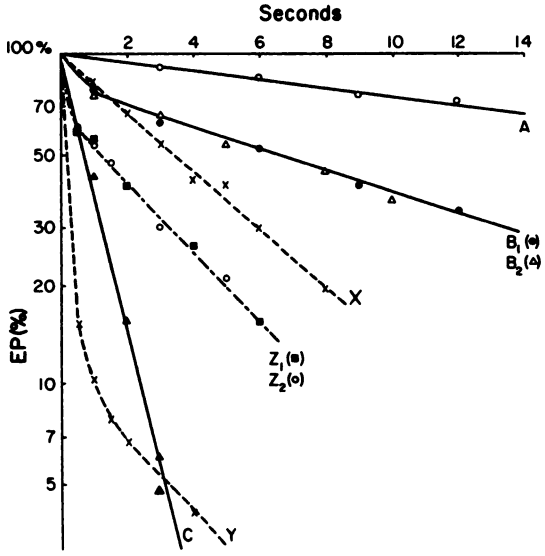


FIG. 1. *Dephosphorylation of eel Na,K-ATPase phosphoprotein at 4°*

Phosphorylation of the eel enzyme was performed at 4° with 2 mM $Mg^{2+}$ and 40 mM Tris-HCl buffer (pH 7.6) in the presence of 1000 mM $Na^+$ and 100 μM [γ-$^{32}$P]ATP (*solid line*) or in the presence of 10 mM $Na^+$ and 16–20 μM [γ-$^{32}$P]ATP (*dashed line*). After 1-sec phosphorylation, the reaction was terminated by the addition of 0.5 volume of 150 mM CDTA containing the same concentration of $Na^+$ without (*line A*, *line X*) or with 6 mM $K^+$ (*line $B_1$*), 10 mM $K^+$ (*line $B_2$*), 3 mM $K^+$ (*line Y*), 0.45 mM ADP (*line C*), 0.15 mM ADP (*line $Z_1$*), or 1.5 mM ADP (*line $Z_2$*). The reaction then was terminated at the indicated intervals and the phosphoprotein was measured as described in the text.

was estimated by extrapolation of the dephosphorylation curves in the presence of $K^+$ or ADP, with the assumption that these two intermediates were not interconverting during the assay. In the eel enzyme, about 90% of the phosphoenzyme formed in the presence of 10 mM $Na^+$ is $E_2P$, and virtually all of the phosphoenzyme formed in the presence of 1000 mM $Na^+$ is $E_1P$.

Under these conditions, the sums of these two phosphoenzymes ranged from 90% to 120%, and a higher concentration of $K^+$ or ADP during the dephosphorylation period did not change the slow phase in the biphasic dephosphorylation curves. In the shark enzyme, $E_1P$ was also predominant in the presence of 1000 mM $Na^+$, as well as $E_2P$ in the presence of 10 mM $Na^+$ (data not shown). These results confirmed the earlier observations of other investigators (10–12).

The presence of intermediate concentrations of $Na^+$ is expected to result in the formation of a phosphoprotein mixture of $E_1P$ and $E_2P$ in the eel enzyme, as reported by Kuriki and Racker (12). However, in the presence of 50 mM $Na^+$, 70–80% of the eel phosphoenzyme was rapidly dephosphorylated in the presence of $K^+$, and more than 90% was rapidly dephosphorylated in the presence of ADP (Fig. 2). Similar results were also obtained in the presence of 100 mM $Na^+$.

In order to study these unexpected characteristics of the eel phosphoenzyme formed in the presence of intermediate concentrations of $Na^+$, we selected the experimental conditions and calculation methods described under Materials and Methods: When the eel enzyme was

[2] In order to eliminate the influence of contaminants in the ADP, which might accelerate the dephosphorylation of the phosphoenzyme, we purified the ADP by DEAE-Sephadex chromatography. There was no detectable difference between the purified and original forms.

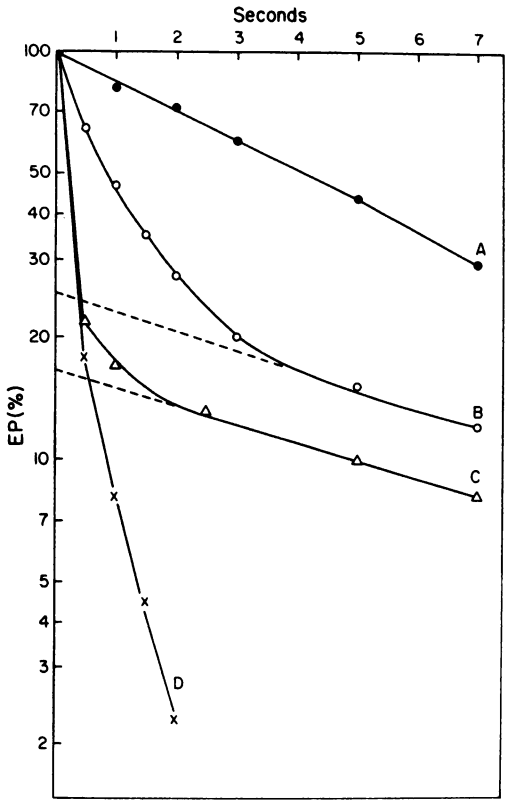


FIG. 2. *Dephosphorylation of eel Na,K-ATPase phosphoprotein formed in the presence of 50 mM $Na^+$ at 4°*

Phosphorylation of the eel enzyme was performed with 50 mM $Na^+$, 2 mM $Mg^{2+}$, 20 μM [$\gamma$-$^{32}$P]ATP, and 40 mM Tris-HCl buffer (pH 7.6). After 1-sec phosphorylation, the reaction was terminated by the addition of 0.5 volume of 150 mM CDTA containing 50 mM $Na^+$ without (*line A*) or with 0.3 mM $K^+$ (*line B*), or with 30 mM $K^+$ (*line C*) or 0.15 mM ADP (*line D*), and the phosphoprotein was measured at the indicated intervals as described in the text.

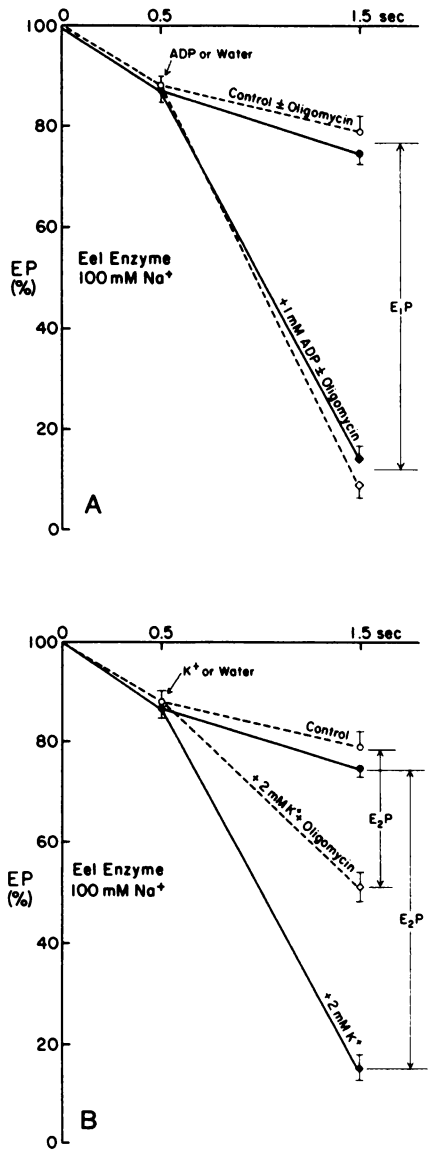


FIG. 3. *Effect of ADP and $K^+$ on dephosphorylation of eel Na,K-ATPase phosphoprotein with or without oligomycin*

The eel enzyme was phosphorylated with approximately 20 μM [$\gamma$-$^{32}$P]ATP, 100 mM $Na^+$, 3 mM $Mg^{2+}$, and 30 mM Tris-HCl buffer (pH 7.6) for 2 sec at 4°. The phosphorylation was terminated at 0 sec by the addition of 50 mM CDTA with (*dashed line*) or without (*solid line*) oligomycin (50 μg/ml); at 0.5 sec, 1 mM ADP (A) or 2 mM $K^+$ (B) was added. The rest of the procedure was the same as that described in Fig. 1.

phosphorylated and dephosphorylated in the presence of 100 mM $Na^+$, about 80% of the phosphoenzyme was ADP-sensitive (i.e., dephosphorylated by 1 mM ADP within 1 sec) (Fig. 3A) and was also $K^+$-sensitive (i.e., dephosphorylated by 2 mM $K^+$ within 1 sec (Fig. 3B). More than 95% of the phosphoenzyme in the eel enzyme was dephosphorylated by the simultaneous addition of 1 mM ADP and 2 mM $K^+$ (data not shown). On the other hand, about 70% of the shark enzyme phosphoenzyme was $K^+$-sensitive and approximately 30% was ADP-sensitive under the same experimental conditions (Fig. 4).

In the shark enzyme, the sum of the percentage of $E_1P$ and $E_2P$ was about 100% in the presence of 100 mM $Na^+$, similar to those in the presence of 10 and 1000 mM $Na^+$. In the eel enzyme, the sum was more than 150%, as shown in Fig. 5, columns 1 and 2. These differences between the two enzymes did not change when 1 mM unlabeled ATP was substituted for 50 mM CDTA (results not shown).

*Effects of oligomycin on dephosphorylation of the phosphoenzyme.* Two different conclusions can be drawn from the unexpectedly large sums of $E_1P$ and $E_2P$ percentages: (a) A significant percentage of the $E_1P$ may convert to $E_2P$ or vice versa during the dephosphorylation period (1 sec). (b) The eel enzyme may produce a new type of phosphoenzyme which is sensitive to both $K^+$ and ADP. To examine these possibilities, the effects of oligomycin on the dephosphorylation of the phosphoenzyme were studied. Several studies have indicated that oligomycin is an inhibitor of the conversion of $E_1P$ to $E_2P$ in Na,K-ATPase (11, 13). No significant changes on the dephosphorylation rates of the shark enzyme in the presence or absence of $K^+$ or ADP were observed when oligomycin (50 μg/ml) was added with CDTA (Fig. 4, *dashed lines*). On the other hand, in the eel enzyme, oligomycin substantially reduced the effects of $K^+$ on the

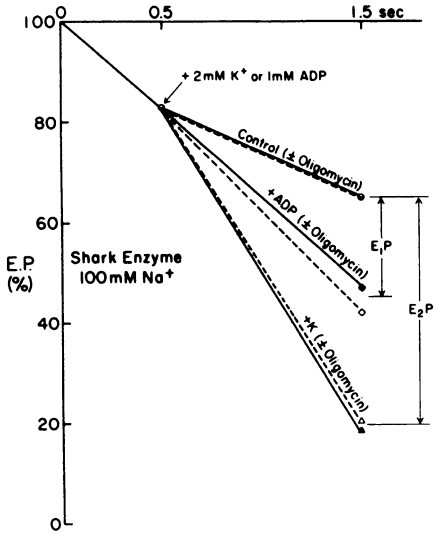


FIG. 4. *Effects of ADP and $K^+$ on the dephosphorylation of shark Na,K-ATPase phosphoprotein with or without oligomycin*

The experimental procedures were similar to those described in Fig. 3 except for the use of shark enzyme.

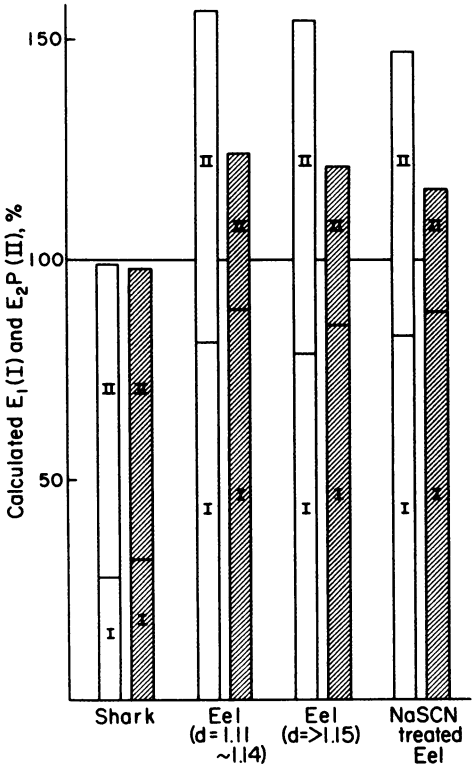


FIG. 5. *Percentages of $E_1P$ and $E_2P$ in phosphoproteins of various Na,K-ATPase preparations*

The experimental procedures and the calculations of percentages of $E_1P$ and $E_2P$ are described in the text, and the *hatched* and *open bars* represent results obtained with and without oligomycin, respectively. The preparations of shark enzyme and eel enzyme (density 1.11–1.14) are described in the text. The other eel enzymes (density >1.15) were separated from the above enzyme (density 1.11–1.14) as the pellet by density gradient centrifugation of eel microsomes (specific activity 2.5 μmoles of $P_i$ per minute per milligram of protein at 30°). The NaSCN-treated eel enzyme was prepared by treating the eel enzyme (density 1.11–1.14) with 1 M NaSCN at room temperature for 60 min (specific activity 6.35 μmoles of $P_i$ per minute per milligram of protein at 30°).

dephosphorylation rate (Fig. 3B, *dashed lines*) and thus reduced the sum of $E_1P$ and $E_2P$ from 160% to 124%; however, when 1 mM $K^+$ was added simultaneously with CDTA and oligomycin, the reduction in the effect of $K^+$ by oligomycin was not as great (data not shown). Perhaps this can be explained by the fact that the inhibition rate of oligomycin is not as great as the reaction rate of $K^+$. On the other hand, the effects of ADP on the dephosphorylation rate were not significantly affected by oligomycin (Fig. 3A, *dashed line*). These effects of oligomycin on the two enzymes were also observed when 1 mM unlabeled ATP was substituted for CDTA.

From these effects of oligomycin, we concluded that $E_1P$ was converted to $E_2P$, then was dephosphorylated by $K^+$ in the eel enzyme, and that the large sums of $E_1P$ and $E_2P$ percentages could be explained without assuming the existence of an ADP- and $K^+$-sensitive phosphoenzyme. Therefore, it is probable that the values of the $E_2P$ percentages obtained in the presence of oligomycin are more accurate.

*Effects of $Na^+$ concentration on the phosphoenzyme.* As described above, a higher $Na^+$ concentration leads to a higher $E_1P$ percentage. The percentages of $E_1P$ and $E_2P$ were examined in various concentrations of $Na^+$, using both eel and shark enzymes. As shown in Fig. 6, when the $Na^+$ concentration decreases from 100 to 10 mM, the eel enzyme $E_1P$ fraction decreased from 81% to 18%, and the $E_2P$ fraction in the presence of oligomycin doubled from 36% to 72%. In addition, the sum of $E_1P$ and $E_2P$ decreased from 160% to 101% even in the absence of oligomycin. Therefore, we conclude that the decrease in the $Na^+$ concentration causes not only an increase in the $E_2P$ fraction but also causes less conversion of $E_1P$ to $E_2P$; in fact, the eel phosphoenzyme obtained in the presence of 10 mM $Na^+$ was similar to the shark phosphoenzyme obtained in the presence of 100 mM $Na^+$.

On the other hand, an increase in the $Na^+$ concentration from 100 to 500 or 700 mM in the shark enzyme led to a higher $E_1P$ percentage, a sum of $E_1P$ and $E_2P$ percentages in excess of 150% in the absence of oligomycin, enhancement of the effects of oligomycin, and more inhibition by oligomycin of the conversion of $E_1P$ to $E_2P$; these characteristics are similar to those of the eel enzyme phosphoenzyme in the presence of 100 mM $Na^+$.

Eel microsomes can be separated into two Na,K-ATPase-rich fractions with densities of 1.11–1.14 and in excess of 1.15 (4). Although the lower density fraction was used extensively in this study, the higher density fraction also produced $E_1P$-rich phosphoenzyme in the presence of 100 mM $Na^+$ and this phosphoenzyme was dephosphorylated by 2 mM $K^+$, whereas oligomycin slowed the dephosphorylation (Fig. 5, column 3).

The eel enzyme is an extremely detergent-sensitive form of Na,K-ATPase (14) and is also rather sensitive to chaotropic reagents. The phosphoenzyme of the Na,K-ATPase treated with 1 M NaSCN (specific activity 4.8 μmoles of $P_i$ per minute per milligram of protein at 30°) was examined (Fig. 5, column 4), and the results obtained were similar to those obtained using the untreated en-

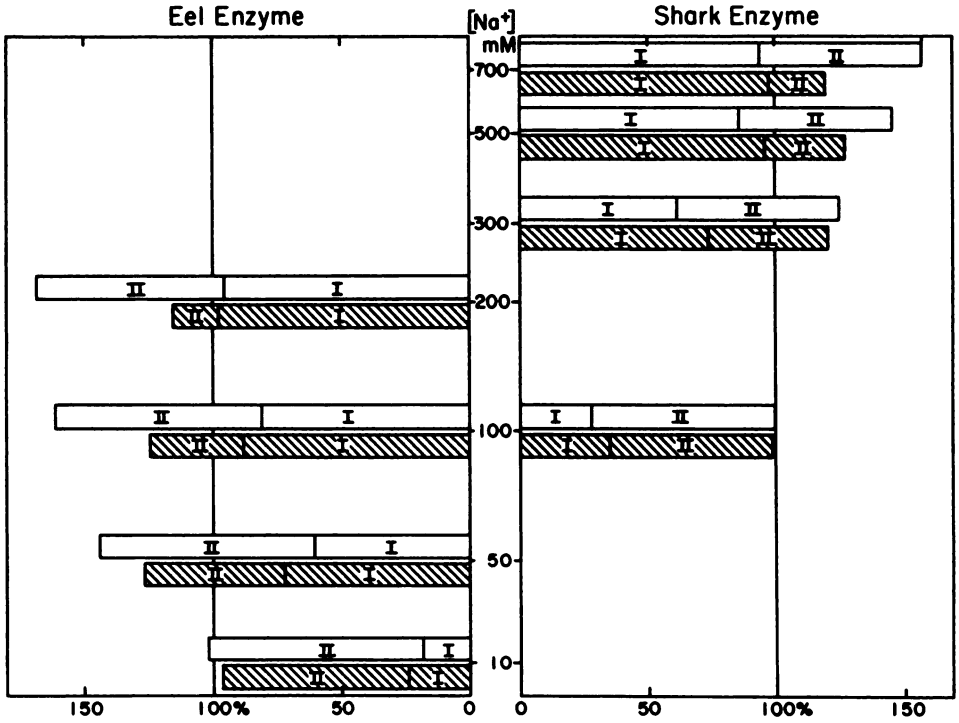


FIG. 6. *Percentage of $E_1P$ and $E_2P$ in phosphorylated Na,K-ATPase in various $Na^+$ concentrations*
The experimental procedures and calculations are similar to those described in Fig. 5.

zyme. The phosphoenzyme of sodium dodecyl sulfate-treated shark enzyme was also examined, but no significant differences were observed between the phosphoenzymes obtained from the treated and untreated enzymes in the presence of 100 mM $Na^+$ (data not shown).

*Dephosphorylation of the phosphoenzyme at 25°.* In the eel enzyme, the dephosphorylation curves of the phosphoenzyme were log linear in the presence or absence of $K^+$ or ADP, as shown in Fig. 7. Although the phosphoprotein formed in the higher $Na^+$ concentration was more sensitive to ADP and less sensitive to $K^+$, these log linear curves were observed for all of the phosphoproteins formed in 10, 100, and 1000 mM $Na^+$.

In the shark enzyme, the dephosphorylation curves of the phosphoenzyme were not log linear, although the phosphoenzyme became more sensitive to both ADP and $K^+$ when the temperature was elevated (Fig. 8). These results suggest not only the interconversion of $E_1P$ to $E_2P$ but also that the reverse change ($E_2P$ to $E_1P$) is accelerated by elevation of temperature.

The effects of oligomycin on dephosphorylation were also examined at 25° to determine whether oligomycin inhibits the conversion of $E_2P$ to $E_1P$. However, this conversion of $E_2P$ to $E_1P$ was not inhibited by oligomycin under the same conditions where the opposite conversion ($E_1P$ to $E_2P$) was inhibited (Fig. 9).

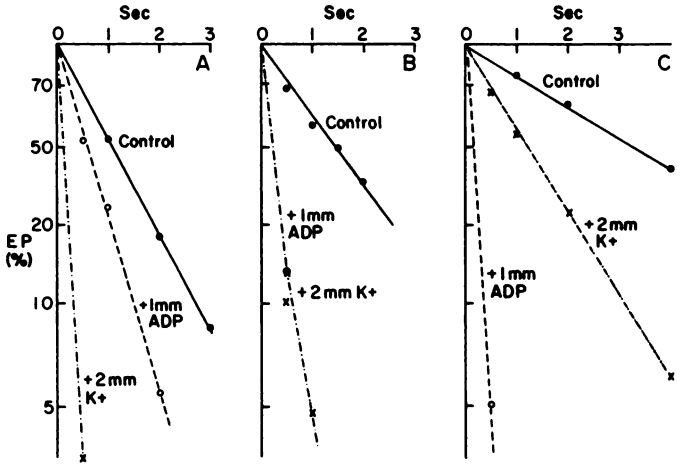


FIG. 7. *Dephosphorylation of eel Na,K-ATPase phosphoprotein at 25°*

Phosphorylation of the eel enzyme was performed at 25° with 20 μM [γ-$^{32}$P]ATP and 40 mM Tris-HCl buffer (pH 7.6) in the presence of 10 mM $Na^+$ and 2 mM $Mg^{2+}$ (A), or 100 mM $Na^+$ and 2 mM $Mg^{2+}$ (B), and of 1000 mM $Na^+$ and 10 mM $Mg^{2+}$ (C). After 1-sec phosphorylation, the reaction was terminated by the addition of 0.5 volume of 150 mM CDTA containing the same concentrations of $Na^+$ without (●) or with 6 mM $K^+$ (×) or 3 mM ADP (○). The phosphoprotein was measured at the indicated intervals.

## DISCUSSION

In the usual reaction scheme of ATP hydrolysis by Na,K-ATPase,

$$E_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow{k_3} E_2$$

$E_1P$ is considered to be a phosphorylated intermediate that is also the precursor of $E_2P$ (3), but the conversion of $E_1P$ to $E_2P$ has seldom been studied for the following reasons. (a) In most enzyme preparations, $E_1P$ is not a major component of the phosphorylated intermediate, even though nearly one-half of the phosphorylated enzyme was $E_1P$ in bovine (15) and rat (16) brain preparations. (b) $E_1P$ is rapidly converted to $E_2P$ in the presence of some ligands, although this fact is generally ignored.

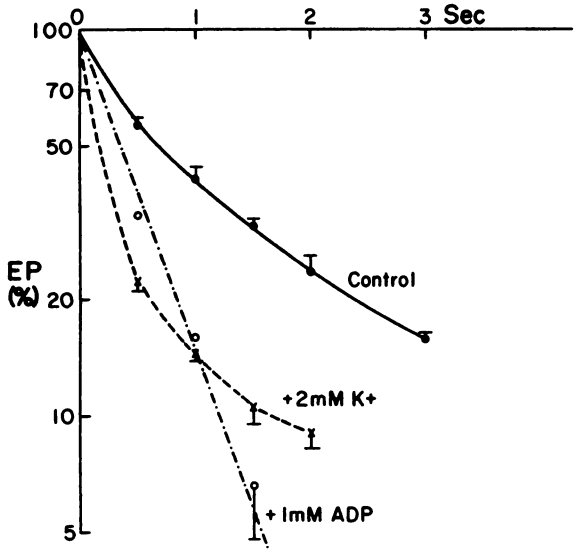


FIG. 8. *Dephosphorylation of shark Na,K-ATPase phosphoprotein at 25°*

The experimental procedures are similar to those described in Fig. 7B. Each value and bar represent an average of triplicate experiments, and the experimental error, respectively.

Here we tried to prevent the conversion of $E_1P$ to $E_2P$ by using oligomycin.

Several papers have concluded that oligomycin is the inhibitor of the conversion of $E_1P$ to $E_2P$ because the $E_1P$ percentage increases in the phosphorylated enzyme when oligomycin is present in the phosphorylating medium (11, 13). This study indicated that oligomycin inhibits the conversion of $E_1P$ to $E_2P$ even when oligomycin is added with the termination of phosphorylation; however, oligomycin neither inhibited nor stimulated the reverse conversion of $E_2P$ to $E_1P$, even at 25°. These results suggest that oligomycin probably interacts only with $E_1P$, and therefore can inhibit only the conversion of $E_1P$ to $E_2P$.

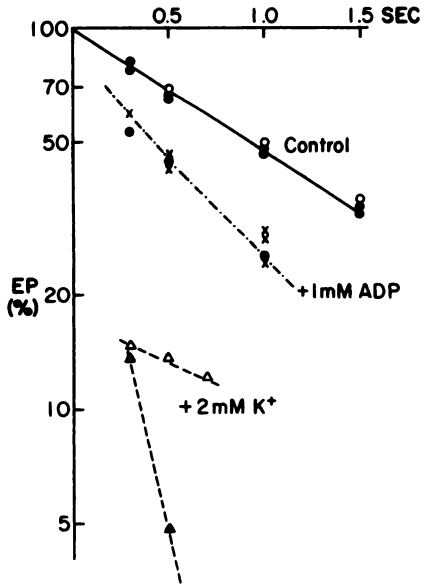


FIG. 9. *Effect of oligomycin on dephosphorylation of eel enzyme at 25°*

Phosphorylation was performed at 25° with approximately 20 μM [γ-$^{32}$P]ATP in the presence of 10 mM $Na^+$, 2 mM $Mg^{2+}$, and 40 mM Tris-HCl buffer (pH 7.6). After 1-sec phosphorylation, the reaction was terminated by the addition of 0.5 volume of a mixture of 150 mM CDTA and 10 mM $Na^+$ without (○, ●) or with 6 mM $K^+$ (△, ▲) or 3 mM ADP (⊗, ×) in the presence (○, △, ⊗) or absence (●, ▲, ×) of oligomycin (150 μg/ml). Other procedures are the same as those described in Fig. 7.

Since oligomycin was not able to inhibit the conversion of $E_1P$ to $E_2P$ rapidly enough in the presence of $K^+$, the $E_1P$ percentages obtained in the presence of oligomycin may not be totally accurate but are certainly close approximations of the actual value. On the basis of these effects of oligomycin on the phosphorylated form of the eel enzyme, we conclude that the eel enzyme always forms the $E_1P$-rich phosphoenzyme (more than 80% of the phosphoenzyme was in the $E_1P$ form) except in low concentrations of $Na^+$ (Fig. 6).

As shown in Fig. 6, the changes in the $E_1P/E_2P$ ratio with or without oligomycin are influenced by the $Na^+$ concentrations in both eel and shark enzymes, and this change in the eel enzyme at $Na^+$ concentrations between 10 and 50 mM is similar to that in the shark enzyme at $Na^+$ concentrations between 100 and 300 mM. It has been suggested that there are two classes of $Na^+$ sites in the Na,K-ATPase arising from the biphasic activation of $Na^+$-dependent ATP hydroysis by $Na^+$ (17, 18). The range of $Na^+$ concentration required to control the $E_1P/E_2P$ ratio is similar to that which is needed to activate the second-phase $Na^+$-dependent ATP hydrolysis. Thus, it may be suggested that the differences in the phosphorylated Na,K-ATPases between the eel and shark enzymes originate from the different affinities of the $Na^+$ site that control the $E_1P$-$E_2P$ equilibrium. This remarkable feature of the eel enzyme should be useful in the study of the $E_1P$ form and the conversion of $E_1P$ to $E_2P$, which is believed to be the step coupled to transport of the inside $Na^+$ ion to the outside.

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