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Evidence for Two Forms of Fluoride-Treated Sodium- and Potassium-Dependent Adenosine Triphosphatase

STANLEY C. PENZOTTI, JR.,[1] AND ELWOOD TITUS

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY

The ($Na^+ + K^+$)-dependent ATPase (EC 3.6.1.3) from rat kidney microsomal fraction was inactivated by 5 mM Mg^{++} and 10 mM F^- after incubation for about 1 hr at pH 8.5, but lower pH values or the presence of 30 mM KCl enhanced the enzyme activity. The inhibited enzyme was not appreciably reactivated by repeated washing of the particles with 0.1 M Tris buffer, pH 7.4, at 0°, or by incubation with buffer at 37°.

Regeneration rates measured in the presence of Na^+ rose with increasing concentrations of this cation and were greatly enhanced by the inclusion of 15 mM Tris-ATP in the incubation medium. Preparations which had been inactivated by F^- in the presence of 30 mM K^+ or had been temporarily exposed to K^+ after prior inactivation recovered activity at about twice the rate of inhibited particles that had never been exposed to K^+. Thus, after 10 min at 37° in 30 mM Na^+ and 15 mM Tris-ATP, particles originally inhibited in the presence of 30 mM K^+ regained 54% of the activity of controls, and preparations inhibited by NaF alone regained 22%. When 30 mM K^+ was added to the solution of 30 mM Na^+ and 15 mM ATP in which regeneration was attempted, no reactivation of the enzyme occurred.

The data suggest that K^+ converts the inactivated enzyme to a K^+ complex that resists reactivation, and that this reaction is associated with a change in conformation of the enzyme. After this change in conformation, the enzyme-potassium complex would appear to have a dissociation constant of the order of 2×10^{-4} M. The affinity for potassium is thus sufficiently low so that washing of the particulate enzyme would be expected to remove this cation. The enzyme is then left in a form that regenerates rapidly with Na^+ and ATP.

INTRODUCTION

It is now well established that the Mg^{++}-requiring, ($Na^+ + K^+$)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) bound within cell membranes is responsible for cation transport (1–3). The mechanism by which the actual transport takes place is still not understood, largely because the enzyme or enzymes responsible resist isolation and purification. However, intermediate steps associated with the ($Na^+ + K^+$)-dependent phosphohydrolase activity can be traced by kinetic studies of the incorporation of ^{32}P from terminally labeled [^{32}P]ATP into membrane phosphoprotein. Fahn *et al.* (4) have proposed that Na^+-dependent, reversible phosphorylation to a high-energy phosphoprotein and a subsequent change, possibly an Mg^{++}-dependent conformational shift to a

[1] Research Associate in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health. Present address, Wyeth Laboratories, Box 8299, Philadelphia, Pa. 19101.

phosphoprotein of lower energy, precede the final K^+-dependent hydrolysis of phosphoprotein. It is also possible that Na^+ and ATP simply put the enzyme into a conformation which has a higher affinity for Mg^{++} (5). Albers *et al.* (6) have pointed out that, whatever the interpretation of the kinetic studies, the vectorial character of the various ion activations of the enzyme in intact cells suggests that the mechanism involves a series of reversible conformational shifts in a system of allosterically linked proteins. Kinetic and other evidence suggestive of the allosteric nature of (Na^+ + K^+)-ATPase has, in fact, appeared in several publications (6–15). It is thus not surprising that a number of efforts should have been made to provide physical evidence for ion-dependent conformational changes (16) and to determine how many proteins in the crude membrane preparations might actually participate in the transport ATPase mechanism (17, 18). Apparently a single protein with a molecular weight of about 93,000 (19) is labeled by the sodium-dependent incorporation of ^{32}P from [^{32}P]ATP and the ouabain-dependent incorporation of ^{32}P from $^{32}P_i$ or from *p*-nitrophenyl phosphate (6, 18, 20, 21). In rat kidney preparations, other proteins are also labeled by diisopropyl [^{32}P]fluorophosphate (17). This labeling is prevented in the presence of ATP, but there is no evidence that the labeled proteins participate in the ATPase reaction mechanism. Although incubation with DFP[2] has been known for several years to inhibit (Na^+ + K^+)-ATPase from various sources (22–24), it was already apparent in the earlier work (24, 25) that labeling by DFP did not correlate with inhibition under several conditions. Since fluoride ion inhibits (Na^+ + K^+)-ATPase (26–28), Yoshida *et al.* (26) have suggested that liberation of this ion from DFP might account for the inhibitory effects. Recent work by Lahiri and Wilson (29) indicates that inhibition by DFP is indeed ascribable to F^-. In this laboratory (Na^+ + K^+)-ATPase from rat kidney was found to be inhibited by incubation with DFP and Mg^{++}. The inhibition was enhanced by K^+ and prevented by ATP, but the effects of these ligands on inactivation did not parallel their effects on the incorporation of phosphorus from [^{32}P]DFP into enzyme protein. Under the conditions of our experiments, fluoride ion from enzyme-independent hydrolysis of DFP was found to enter the reaction system at 4.2 mmoles/hr, a rate sufficient to account for most, if not all, of the inhibition. It was thus evident that studies of the mechanism of fluoride inhibition would be more informative than the continued use of DFP. During such studies, it has become apparent that cation-dependent conformations existing at the time of inactivation are preserved in the fluoride-treated enzyme. Even though the fluoride form of the enzyme remains inactive through repeated washings, K^+-dependent changes can be demonstrated to occur in the inactive state.

[2] The abbreviation used is: DFP, diisopropyl fluorophosphate.

MATERIALS AND METHODS

Rat kidney microsomes were prepared according to the method of Skou (30) and treated with NaI solution according to Nakao *et al.* (31). The final concentration of enzyme ranged from 5 to 10 mg/ml with an (Na^+ + K^+)-ATPase activity of 80–130 μmoles of P_i per milligram per hour and an Mg^{++}-ATPase activity of 2–6 μmoles of P_i per milligram per hour. The enzyme was frozen at −20° in 0.5-ml aliquots and used for up to 2 months with little loss in activity.

Incubations with DFP or F^- were carried out at 37°. The incubation tubes contained in a 1.0-ml volume, 0.1 M Tris-HCl (either pH 8.5 or, in some experiments, pH 7.4), 0.7–1.0 mg of protein, and 5 mM $MgCl_2$, except when Mg^{++} was studied as a variable. After incubation the reaction was stopped by the addition of 5 ml of cold Tris-HCl (0.1 M, pH 7.4). The enzyme was centrifuged, and the pellet was resuspended in Tris and again centrifuged. The enzyme was then diluted in deionized H_2O, and aliquots were added to the ATPase reaction mixture. The size of the aliquot was chosen so that no more than 20% of the ATP in the incubation mixture would be hydrolyzed during the standard 4-min incubation period, since P_i release is linear with time within this limit. In prep-

arations expected to have low activity, 0.7–1.0 mg of protein was usually used. The ATPase assays were carried out at 37° using ^{32}P-labeled ATP according to the method of Chignell *et al.* (32), with the following modifications. The volume of the assay system was 1.0 ml, and it contained 2 mM [^{32}P]ATP, 5 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.4), and, when used, 120 mM NaCl and 30 mM KCl. Each determination of liberated P_i was carried out in triplicate. The reaction was stopped by addition of a mixture containing 0.2 ml of 10 N H_2SO_4, 0.2 ml of 10% ammonnium molybdate, and 0.1 ml of 0.1 M silicotungstic acid. With the concentration of K^+ used, regeneration of fluoride-inhibited activity during the assay period was negligible. Fluoride consistently inhibited the enzyme to within 2–3% of controls. A 3% increment over the baseline of Mg^{++}-dependent activity is not statistically significant. Protein was measured by the method of Lowry *et al.* (33). $(Na^+ + K^+)$-ATPase activity was calculated as the increment in activity observed when Mg^{++} was replaced by a mixture of Mg^{++}, Na^+, and K^+ as the activating cations. Studies of the rate of formation of F^- from DFP were carried out in a Radiometer pH-stat at pH 8.5 by automatic titration of newly formed HF with 0.01 M 2-amino-2-methyl-1-propanol.

For the recovery experiments, the enzyme was first treated with 5.0 mM NaF with or without 30 mM KCl, as in the preliminary inhibition studies. The incubations were stopped by the addition of 5 ml of cold Tris-HCl as before. The enzyme was centrifuged and washed once. After centrifugation and removal of the final wash solution, 0.1 mM Tris containing 5 mM $MgCl_2$, together with NaCl, ATP, and KCl as needed, was added to the tube containing the pellet. This was quickly suspended in the reaction mixture with the aid of a Teflon plunger, and the tubes were incubated at 37° for the appropriate intervals. The incubations were terminated as before with 5 ml of cold Tris. The microsomes were washed twice with Tris, and the pellets were finally suspended in deionized H_2O for assay of ATPase activity.

Diisopropyl [^{32}P]fluorophosphate was supplied by Amersham/Searle at a specific activity of 80 mCi/mmole. Unlabeled DFP was purchased from Calbiochem. Terminally labeled [^{32}P]ATP was kindly supplied by Mr. George Koval.

RESULTS

The time course of NaF inhibition was examined, with the results shown in Fig. 1. The enzyme activity decreased with time in the presence of 5 mM NaF, and the rate of inhibition was dramatically increased by the addition of K^+. The activities of samples incubated with K^+ alone were identical with those of the controls. At the end of 60 min. there was essentially no difference between the two methods of inhibition. The enzyme without F^- spontaneously lost about 30% of the original activity during the incubation at 37° for 60 min. It appears from these data that the inhibition by F^- cannot be described as a simple pseudo-first-order reaction. Lahiri and Wilson (29) postulated that their guinea pig kidney enzyme might contain two components, one of which was resistant to attack by F^-. The reason for the decrease with time of the apparent rate constant for inactivation is unknown. Similar effects of potassium were seen when percentage inhibition was plotted against increasing NaF concentrations (Fig. 2). Figure 3 shows that, as in the experiments with DFP, disodium ATP in sufficient concentrations can protect the enzyme from inhibition by F^-. In the absence of K^+, the activities with high ATP

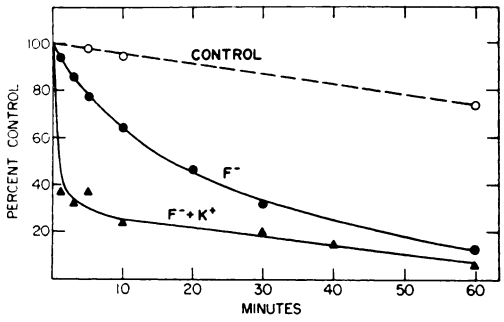

FIG. 1. *Time course of inhibition of* $(Na^+ + K^+)$-*ATPase by NaF*

Incubations with 5 mM DFP were carried out at pH 8.4 as described in the text. Points are the means of two separate determinations of $(Na^+ + K^+)$-ATPase activity in rat kidney microsomes incubated without NaF (○), with 5 mM NaF (●), and with 5 mM NaF plus 30 mM KCl (▲).

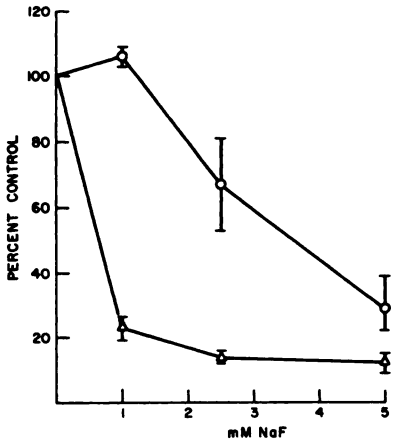

FIG. 2. *Effects of increasing concentrations of NaF on $(Na^+ + K^+)$-ATPase activity of rat kidney microsomes*

Incubations were carried out for 60 min as in Fig. 1 in the presence of various concentrations of NaF (○), or NaF plus 30 mM KCl (△). Points are the means ± the range of values, from three experiments, of the enzymatic activity remaining after incubation.

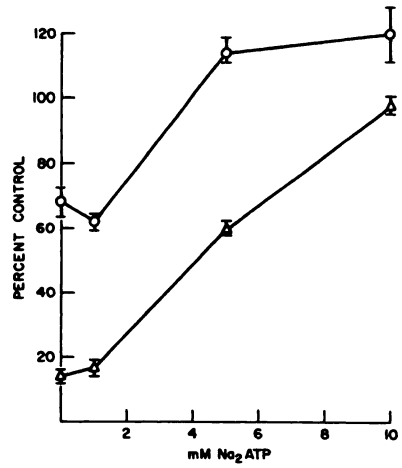

FIG. 3. *Effect of disodium ATP on inhibition of $(Na^+ + K^+)$-ATPase by 5 mM NaF*

Incubations were carried out as in Fig. 4, but with varied concentrations of disodium ATP present during exposure to 5 mM NaF (○), or 5 mM NaF plus 30 mM KCl (△). Points are the means ± range of values, from three experiments, of the enzymatic activity remaining after incubation.

concentrations were slightly greater than 100%, probably because disodium ATP help to stabilize the enzyme during incubation at 37°.

Protection against the inactivating effects of F^- could also be obtained with Na^+ alone, but at higher concentrations than those afforded by the addition of disodium ATP. Thus, in experiments with the same batch of enzyme used in Fig. 3 and under the same conditions, the addition of sufficient NaCl to give a total Na^+ concentration of 15 mM did not significantly increase the $(Na^+ + K^+)$-ATPase surviving after exposure to 5 mM F^-. Concentrations of 25 mM and 100 mM Na^+, however, gave 41% and 84%, respectively,

The inhibition by NaF is known to be affected by Mg^{++} and pH (26–29) as well as by KCl. In the rat kidney system at lower pH values, i.e., 5.5 and 6.5, inhibition was essentially complete in 5 min with or without K^+. As the pH was increased, the rate of inhibition decreased, until at pH 9 there was practically no inhibition by NaF alone, whereas the reaction with NaF and KCl was still inhibited about 70% (Fig. 4).

Mg^{++} was required for the inhibitory effects of F^- (Fig. 5). The concentration of Mg^{++} necessary for half-maximal inhibition was approximately 0.25 mM, which is close to the value of 0.3 mM found by Lahiri and Wilson (29). Inhibition by F^- could not be expressed as a simple hyperbolic function of Mg^{++} concentration, since there appeared to be a threshold level of Mg^{++} which had to be surmounted before inhibition occurred. Some nonspecific adsorption of Mg^{++} to micro-

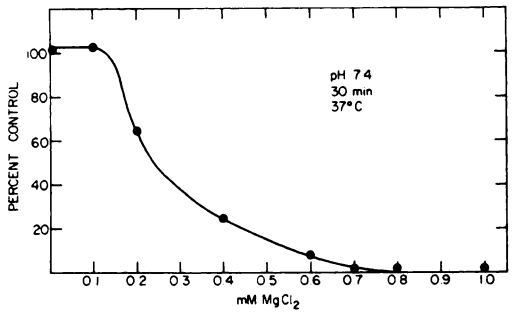

FIG. 4. *Effect of $MgCl_2$ on inhibition of $(Na^+ + K^+)$-ATPase by 5 mM NaF*

Incubations were carried out at 37° for 30 min with 5 mM NaF and variable concentrations of $MgCl_2$. Points represent the means of two determinations of enzyme activity remaining after incubation, as described in the text.

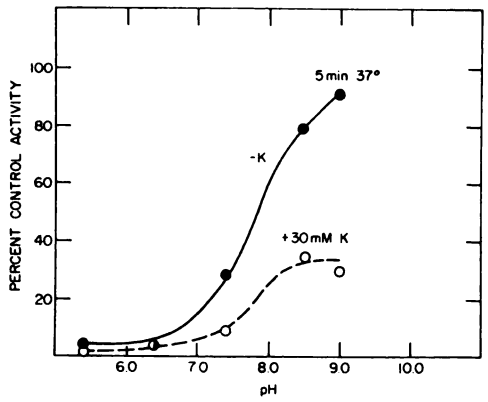

FIG. 5. *Effect of pH on rate of inhibition of $(Na^+ + K^+)$-ATPase by 5 mM NaF*

Incubations were carried out at 37° for 5 min in 5 mM NaF (●) or 5 mM NaF plus 30 mM KCl (○). Points are the means of duplicate determinations of enzyme activity remaining after incubation.

somal phospholipid may have accounted for this.

The inhibition by F^- was thought to be irreversible (26), but later observations indicated a slow recovery (28, 29). In this laboratory, washing with Tris buffer caused very little recovery. The experiments of Fig. 4 suggest that the inhibited form of the enzyme is probably a complex including magnesium and fluoride ions. Preparations that had been extensively inactivated by 30 min of exposure to NaF at pH 8.4 were therefore washed with EDTA to test whether removal of magnesium could dissociate the complex and restore normal activity. At 0° there was no effect of EDTA, and even at 37° the regeneration of enzymatic activity was minimal (Table 1).

Since the experiments described above as well as those of other laboratories (28, 29) indicated that disodium ATP protects against fluoride inhibition, it was decided to test whether disodium ATP might regenerate enzymatic activity. As shown in Table 2, incubation for 30 min with disodium ATP did indeed reverse the inhibition, the degree of reversal increasing with disodium ATP concentration. Moreover, at all concentrations of disodium ATP, the enzyme which had been inhibited in the presence of K^+ recovered to a greater degree than enzyme which had been treated solely with NaF.

TABLE 1

Effect of Tris-EDTA on recovery of $(Na^+ + K^+)$-ATPase activity after inhibition by F^-

Suspensions containing 1 mg of protein per milliliter, 5 mM $MgCl_2$, and 5 mM NaF in 0.1 M Tris, pH 8.4, were incubated for 30 min at 37°. When used, KCl was present at a concentration of 30 mM. The reaction was stopped by dilution as described in the text, and the washed pellets were subjected to a second 30-min incubation in 0.1 M Tris, pH 7.4, with or without EDTA. The reaction was stopped by dilution, and the pellets were washed before assay as described elsewhere. Control samples were carried through incubations in the Tris buffers without other additions. Experiments at 0° and 37° were performed with different aliquots of enzyme. Each value is the mean of two determinations.

KCl in first incubation	Second incubation		Percentage of control activity
	Temperature	Tris-EDTA	
mM		*mM*	
0	0°	10	31.8
0	0°	0	31.4
30	0°	10	13.9
30	0°	0	14.4
0	37°	10	39.5
0	37°	0	25.3
30	37°	10	25.8
30	37°	0	15.6

This occurred even though both forms were inhibited to the same degree before addition of disodium ATP. When 30 mM K^+ was present during treatment of the previously inhibited enzyme with disodium ATP, the recovery was less than that observed in 0.1 M Tris buffer. The recovery of the enzyme inhibited by NaF and that inhibited by NaF plus K^+ was a linear function of time through at least 10 min (experiment 2 of Table 2). Brief exposure of the inactivated enzyme to K^+ converted the slowly recovering form to the more rapidly reactive form (experiment 3 of Table 2).

Since replacement of disodium ATP by 15 mM Tris-ATP markedly decreased the recovery rate of the inhibited enzyme, it appeared that Na^+ was required for recovery. In accord with this view, increasing concentrations of Na^+ alone accelerated the recovery of both forms of the inhibited en-

TABLE 2

Recovery of ($Na^+ + K^+$)-ATPase activity after inactivation with F^-

The enzyme was inactivated in the first incubation, which was carried out at 37° in 5 mM NaF for 30 min as described in the experiments of Table 1, but at pH 7.4. The reaction was stopped by dilution as described in the text, and the washed particles were resuspended in 0.1 M Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and various concentrations of disodium ATP as shown. KCl, when included in either the first or second incubation, was present at a concentration of 30 mM. The reaction was stopped by dilution at the times shown, and the particles were washed and assayed as described elsewhere. Values are the means of two determinations unless otherwise indicated. Enzyme activities are expressed as a percentage of the activity of a control carried through identical operations except that fluoride was omitted.

Expt.	KCl in first incubation	Second incubation		Activity recovered after second incubation for			
		Disodium ATP	KCl	0 min	5 min	10 min	30 min
	mM	*mM*		*% control*			
1	0	0	0				2.5
	30	0	0				0
	0	5	0				34.1
	30	5	0				17.9
	0	10	0				68.8
	30	10	0				48.6
	0	15	0				90.9
	30	15	0				59.2
	0	5	30				5.3
	30	5	30				0
	0	15	30				7.5
	30	15	30				3.9
2	0	15	0	2.4	9.9	22.6	
	0	15	0		11.5	24.6	
	30	15	0	2.6	25.9	47.4	
	0	0	30			3.5	
3[a]	0	15	0			26.9 ± 1.2[b]	
	30	15	0			48.6 ± 2.6	
	0	15	30[a]			46.5 ± 6.1	
	0	0	0			3.5	
	30	0	0			6.2	
	0	0	30[a]			2.6	

[a] In these experiments the particles after the first incubation were suspended for 2 min in 30 mM KCl and 0.1 M Tris, pH 7.4. The reaction was stopped, and the recovered washed particles were then incubated for 10 min as usual in a medium without KCl. Standard errors were determined for four experiments, each carried out with triplicate determinations of ATPase activity.

[b] Mean ± standard error.

zyme (Fig. 6). The difference between the recovery rates of the two forms in the absence of ATP, as shown in the lower curves of Fig. 6, is small, and there are insufficient data to establish a statistically significant separation of the two curves. In the presence of ATP, however, the difference between the two forms becomes readily apparent. It can be seen from Fig. 6 that the dependence on sodium concentration cannot be described by the usual simple hyperbolic relationship. Although the curves in Fig. 6 might appear at first glance to be sigmoidal, the data did not give a linear Hill plot. If it is assumed that no reaction occurred until a threshold concentration of 10 mM Na^+ had been exceeded, the recovery rates of the inhibited enzyme in the presence of ATP can be

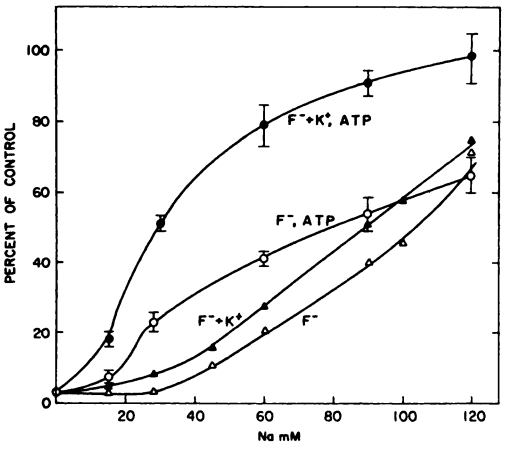

FIG. 6. *Effect of Na^+ and ATP on recovery of (Na^+ + K^+)-ATPase activity after inhibition with NaF*

The enzyme was incubated for 30 min at 37° and pH 7.4 in 5 mM NaF (○, △) or 5 mM NaF plus 30 mM Kcl (●, ▲). The reaction was stopped by dilution, and the centrifuged particles were washed as described in the text. The preparations were incubated for a further 10 min at pH 7.4 and 37° in various concentrations of Na^+ (△, ▲) or Na^+ plus 15 mM Tris-ATP (○, ●). Points are the means of two determinations (△, ▲) or the means ± standard errors of five determinations (○, ●) of enzyme activity. Activities are expressed as a percentage of control preparations carried through identical treatment except for the omission of fluoride.

accommodated reasonably well by the conventional Lineweaver-Burk double-reciprocal plot (Fig. 7). The apparent K_m values for Na^+ calculated from the data of Fig. 7 are 54.1 mM for the enzyme that was inactivated by NaF alone and 25.4 mM for the enzyme exposed to K^+ during inactivation.

The data presented thus far suggest two conformations for the fluoride-inhibited enzyme, but the response to K^+ becomes paradoxical if the forms of the enzyme are limited to only two. Thus, although the slowly recovering form of the enzyme could be converted to a rapidly regenerating form by temporary exposure to K^+, recovery was blocked in the presence of K^+. It therefore seems preferable to assume that this conversion proceeds by way of a K^+-containing intermediate which is incapable of regenerating enzymatic activity. The affinity of this form for K^+, however, would be relatively low, since washing with buffer is sufficient to yield the rapidly reactivatable conformation. If this model is correct, it should be possible to determine a dissociation constant for the complex between the fast-reacting form and K^+ by measuring the effect of incremental additions of K^+ on the sodium-dependent rate of regeneration. Such additions of K^+ are shown in Fig. 8 to slow the rate of regeneration. Measurements were made in both low (15 mM) and high (120 mM) concentrations of Na^+ with the expectation that the apparent dissociation constants for the K^+ complex might be a function of the Na^+ present. The concentrations of K^+ required for half-maximal inhibition, however, were essentially the same in both experiments.

DISCUSSION

Several membrane-bound enzyme systems that require magnesium ion appear capable of forming stable complexes with fluoride ion. The consequences of this complexation can be quite different with different preparations. Adenyl cyclase of brain (34) can be converted by fluoride to a long-lasting, active

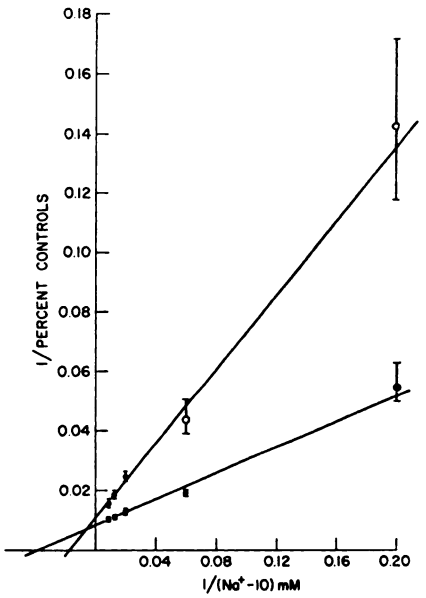

FIG. 7. *Lineweaver-Burk plot of data from Fig. 6*

Reciprocals of enzyme activity were plotted against the reciprocal of Na^+ concentration (10 mM). The symbols (○, ●) are defined in Fig. 6.

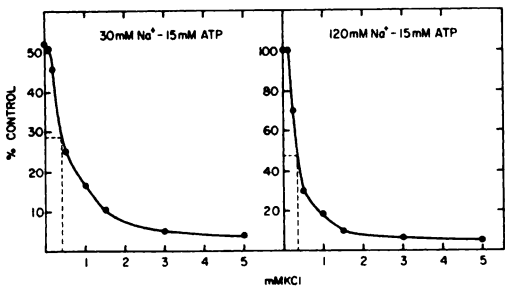

FIG. 8. *Effect of K^+ on Na^+-dependent rate of recovery of (Na^+ + K^+)-ATPase after inhibition by NaF plus KCL*

The enzyme was incubated with 5 mM NaF plus 30 mM KCl for 30 min at 37° and pH 7.4. The reaction was stopped by dilution, and the centrifuged particles were washed as in previous experiments. The particles were then incubated at 37° for 10 min in various concentrations of KCl containing either 30 mM Na^+ and 15 mM ATP or 120 mM Na^+ and 15 mM ATP. Points are the means of duplicate determinations of enzymatic activity remaining after the 10-min incubation. Dashed lines indicate the concentration of K^+ required for half-maximal inhibition.

form that no longer requires hormonal activation. On the other hand, treatment of the (Na^+ + K^+)-ATPase with fluoride converts it to an inactive form that remains inert throughout repeated washings. The rapid release of fluoride from DFP under conditions of pH and temperature commonly used for enzyme incubations has been found sufficient to explain the inhibition of (Na^+ + K^+)-ATPase by DFP (29). The requirement for Mg^{++}, the enhancement by K^+, and the preventive effects of ATP all apply to the inhibitory effects of fluoride as well as to those earlier reported for DFP. These observations unfortunately render invalid earlier efforts (17, 22–24) to identify membranous proteins associated with the transport ATPase by introduction of ^{32}P from DFP into sites protectable by ATP. It now appears, however, that the fluoride ion itself may offer a useful tool for eliciting information about the nature of the ATPase, since recovery from the inactive state occurs at easily measurable rates which differ for different ion-dependent conformations.

The actual mechanism by which fluoride inhibits the enzyme is still unknown. Mg^{++} is required for the inhibition as well as for the normal enzymatic activity (Fig. 5) (27, 28). Since the extent of inhibition increases with Mg^{++} concentration, removal of this ion from the enzyme into soluble MgF_2 seems less likely than the formation of an inactive enzyme-magnesium-fluoride complex. The magnesium in the complex is presumably very stably bound, since incubation of the inactivated enzyme with EDTA gave no more than 15% reactivation. Removal of the activating Mg^{++} as a salt of enzymatically generated fluorophosphate has been shown to be the mechanism by which F^- inhibits enolase (35), and is a plausible basis for these observations. There is also the possibility that the stoichiometric quantities of fluorophosphate formed at the active center and remaining bound to the enzyme would be inhibitory. All attempts to detect radioactive fluorophosphate by paper chromatography of material recovered from incubation media or from enzyme inactivated in the presence of small amounts of [^{32}P]ATP or $^{32}P_i$ have been unsuccessful.

The inhibition by F^- was thought at first to be irreversible (26), but in later reports was considered to be slowly reversible (28, 29). In our experiments it was found that the inhibition could be significantly reversed in a short time if the enzyme was incubated at 37° with disodium ATP.

In the presence of a fixed concentration of ATP, the rate of regeneration was a function of Na^+ concentration. The rather unusual curves depicting the relationship between reactivation rate and Na^+ concentration (Fig. 6) could not be accommodated by a Hill plot, and were clearly not the simple hyperbolae to be expected from the Michaelis-Menten formulation. They could, however, be accommodated in this function if it were assumed that a threshold concentration of 10 mM Na^+ had to be exceeded before this ion began to activate the recovery process. Kinetic analysis of the regeneration curves in the absence of ATP was not attempted, because a statistically significant difference between the two curves could not be proved. Even in the presence of ATP, the extent of recovery in 10 min becomes unreliable as a measure of rate when recovery approaches 100%. Statements

about recovery mechanism are necessarily somewhat equivocal. If, however, the Michaelis-Menten model obtains, the K_m for Na^+ in the regeneration process would be approximately 54.1 mM for the enzyme not exposed to K^+ and 25.4 mM for that treated with K^+. The latter figure is in good agreement with a value of 22.7 mM observed in a rat liver microsomal preparation (not treated with NaI) by Chignell and Titus (36). If some binding site for sodium must be saturated before the ion can interact with the enzyme, it is not clear what this site might be.

A surprising finding was that the enzyme inhibited by F^- in the presence of K^+ was more responsive to the effects of Na^+ than was enzyme inhibited by F^- alone. This was true, however, only for those preparations that had been washed after inhibition. If K^+ remained present, disodium ATP was unable to promote recovery. These observations could be explained by the scheme shown in Fig. 9, in which the conversion of the Na^+-dependent conformation of the fluoride-inactivated enzyme to an easily dissociable K^+ complex is associated with a conformational change. Although the K^+ complex itself is depicted as difficult to restore to normal activity, washing out of the cation leaves the enzyme in its more rapidly regenerated conformation. Such a model predicts that the binding of the K^+ in the complex should be relatively weak, and the dissociation constants of approximately 2×10^{-4} M suggested by the data of Fig. 8 are in accord with this idea. Some of the features of this scheme for the fluoride-inhibited enzyme are reminiscent of the brain enzyme studied by Nørby and Jensen (37). In their studies, the dissociation constant of the enzyme-ATP complex increased from 0.177 μM to a maximum of 0.604 μM as the concentration of K^+ was increased. There is thus a precedent for a K^+-dependent decrease in affinity for ATP, and it is possible that a similar effect is responsible for the blockade by K^+ of ATP-dependent

Nagai *et al.* (14) have found that the fluorescence of anilinonaphthalenesulfonic acid was changed when K^+ was added to a system containing the substance bound to the microsomal ATPase from guinea pig cerebral cortex. They interpreted these changes as evidence for a conformational shift caused by the addition of K^+. Akera and Brody (38) showed that the ouabain-binding site in an enzyme prepared from rat brain was exposed only when the enzyme was in a particular conformation, and that K^+ would induce a conformational transition in which the binding site was much less accessible. In the latter experiments, the form of the enzyme which normally released bound ouabain rapidly was found to release the drug very slowly when K^+ was present in the medium. Allen *et al.* (39) have also shown that the rates of dissociation of ATPase-glycoside complexes reflect the conditions under which they were formed. K^+ stabilized the less stable complex, i.e., that prepared in the presence of ATP, Mg^{++}, and Na^+. Experiments on the effects of temporary exposure to K^+ on the release of bound ouabain were not carried out. If such temporary exposure to K^+ should convert the slowly releasing form of the ATPase-ouabain complex to the rapidly releasing form, the ion-dependent conformational changes postulated by these workers could be analogous to those reported here.

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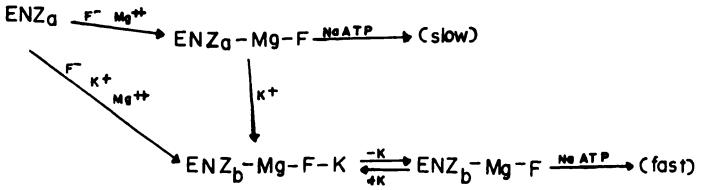

FIG. 9. *Proposed scheme of ion-dependent conformational changes of fluoride-inhibited* (Na^+ + K^+)-*ATPase*

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