TRANSPORT ATPase: THIMEROSAL INHIBITS THE Na^++K^+-DEPENDENT ATPase ACTIVITY WITHOUT DIMINISHING THE Na^+-DEPENDENT ATPase ACTIVITY

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A guinea pig kidney membrane preparation was incubated with thimerosal and then thoroughly washed. Comparison of the properties of the native and the modified membranes showed that (a) Na^++K^+-dependent activity is substantially inhibited by thimerosal; (b) thimerosal does not diminish Na^+-dependent ATPase activity; and (c) the thimerosal treated enzyme, like the native enzyme, is phosphorylated in the presence of Na^+ and ATP, and dephosphorylated upon the addition of K^+. It is suggested that thimerosal does not affect the binding of ATP to the high-affinity catalytic site, but that it blocks the binding of ATP to a low affinity modifying site the occupation of which is essential for the dissociation of the stable K^+-dephosphoenzyme and the recycling of the enzyme.

It has been observed repeatedly that membrane preparations containing Na^++K^+-dependent ATPase activity (EC 3.6.1.3) also catalyze a Na^+-dependent ATPase activity (1-7). In the presence of Na^+ plus K^+, the K_m for ATP is several orders of magnitude higher than the K_m in the presence of Na^+ alone (2,3,5); and when sufficiently low concentrations of substrate are used an inhibitory effect of K^+ on Na^+-dependent activity is obtained (3,5). Some investigators have interpreted these observations to mean that there are two different enzymatic sites: One involved in Na^++K^+-dependent activity, and the other catalyzing a Na^+-dependent and K^+-inhibited activity (3,5). Since both sites are inhibited by cardiac glycosides, different physiological transport functions for the sites have been suggested (3,5). Others have provided evidence, or arguments, in support of the identity of the active sites of the two operationally distinct hydrolytic activities (6-8). The clarification of

the relationship between these activities is of obvious importance to the ultimate understanding of the reaction mechanism of the transport enzyme. Here, we report that a novel modifier of the enzyme inhibits one of these activities without affecting the other. A summary of certain other pertinent properties of the modified enzyme are also presented. Our findings provide strong support for the existence of two distinct ATP binding sites on the enzyme. They do not prove, however, that both sites are catalytic.

Methods

γ-Labeled [^{32}P]ATP was obtained from New England Nuclear (Boston, Mass). ATP, and thimerosal (ethylmercurithiosalicylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). The source of enzyme was a guinea pig kidney membrane preparation (7). The modified enzyme was obtained by incubating these membranes (100 μg protein/ml), at 37° in a solution containing 10^{-4} M thimerosal and 50 mM Tris-HCl (pH 7.4). After 10 min., the membranes were collected by centrifugation at 35,000 Xg, and washed repeatedly with a solution containing 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8).

All media for the assay of ATPase activity contained 3 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4); and the indicated concentrations of the labeled ATP, NaCl, and KCl. The released P_i was converted to phosphomolybdate, extracted into isobutanol, and counted (5). In all assays the reaction time and the amount of enzyme were so chosen to obtain no more than 15% utilization of the substrate. It was shown that under these conditions the amount of released P_i was a linear function of time. The determination of ^{32}P-labeled phosphoenzyme was done as described before (9).

Results

Table 1 shows the ATPase activities of the native enzyme and the thimerosal-treated enzyme at two concentrations of ATP (2 mM and 0.5 μM). In agreement with previous observations (3,5), at the higher substrate concentration substantial stimulation of the ATPase activity of the native enzyme is dependent on the simultaneous presence of Na^+ and K^+; whereas at the lower substrate concentration a Na^+-dependent activity that is inhibited by K^+ is observed. As evident from the data, this Na^+-dependent activity remains unaltered in the thimerosal-treated enzyme. The Na^++K^+-dependent activity of the modified enzyme is, however, reduced to about 20% of the activity of the native enzyme. (When various enzyme preparations are treated with thimerosal under the conditions described in Methods, 70-95% of the Na^++K^+-dependent activity is inhibited without any inhibition of Na^+-dependent activity. By increasing the

Table 1. Effects of thimerosal on the Na^++K^+-dependent and the Na^+-dependent ATPase activities of a guinea pig kidney membrane preparation. The control enzyme and the thimerosal-treated enzyme were prepared and assayed as described in Methods. In addition, the thimerosal-treated enzyme was preincubated at 37° with 1 mM dithiothreitol for 5 minutes, and then assayed. Concentration of Na^+ was 100 mM, and that of K^+ was 25 mM.

Enzyme	ATP Concentration	Activity (μmoles Pi/mg/hr) in the presence of: Mg^{2+}	Mg^{2+}+Na^+	Mg^{2+}+Na^++K^+
Control	2 mM	10.0	11.0	95.2
	0.5 μM	0.43	1.71	1.06
Thimerosal-treated	2 mM	10.5	12.8	29.8
	0.5 μM	0.50	1.83	0.52
Thimerosal-treated + dithiothreitol	2 mM	11.0	13.5	108.5

concentration of thimerosal, complete inhibition of the former activity is obtained at the expense of partial inhibition of the latter.)

The data of Table 1 also show that the Na^++K^+-dependent activity of the modified enzyme is restored when the enzyme is preincubated with 2,3-dithiothreitol. This suggests that a sulfhydryl group may be involved in the reaction of the organomercurial with the enzyme.

The concentrations of Na^+ and K^+ that are used in the experiments of Table 1 are optimal for the assay of Na^++K^+-dependent activity of the native enzyme at 2 mM ATP. Since it seemed possible that the apparent inhibition of the Na^++K^+-dependent activity by thimerosal may be due to changes in optimal concentrations of the activating ions, the effects of a wide variety of Na^+:K^+ concentration ratios on the activity of the thimerosal-treated enzyme were studied. Na^++K^+-dependent activity, approaching that of the native enzyme, could not be detected under any condition.

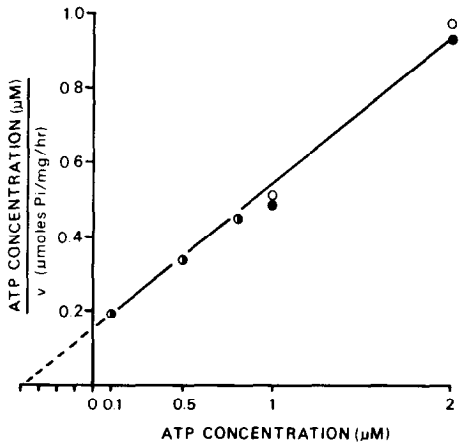

Fig. 1. Effect of substrate concentration on Na^+-ATPase activities of the control (O) and the thimerosal-treated (●) enzymes. Na^+ concentration was 100 mM. Other conditions are described in Methods.

Although the data of Table 1 show no diminution in the Na^+-dependent activity after thimerosal treatment, it was of interest to know if the kinetic parameters of this activity are in any way altered. The data of Fig. 1 show that K_m and V_{max} values of the native enzyme and the thimerosal-treated enzyme are identical.

In the experiments of Fig. 2 the effects of Na^+ and K^+ on the reaction of ATP with the native enzyme and the thimerosal-treated enzyme are compared. The following are evident from the data: 1. The thimerosal-treated enzyme, like the native enzyme, is phosphorylated in the presence of Na^+ and ATP. 2. K^+ has similar activating effects on the discharge of both phosphoenzymes.

Discussion

At first glance the data of Table 1 and Fig. 1 may seem to support the existence of two distinct catalytic sites capable of independent operation, as suggested by Czerwinski et al. (3), and by Neufeld and Levy (5). One could simply suggest that thimerosal is a selective inhibitor of one of these sites. While this hypothesis remains a possibility, it should be recalled that the findings of Post et al. and Siegel and Goodwin, (7,8) have already

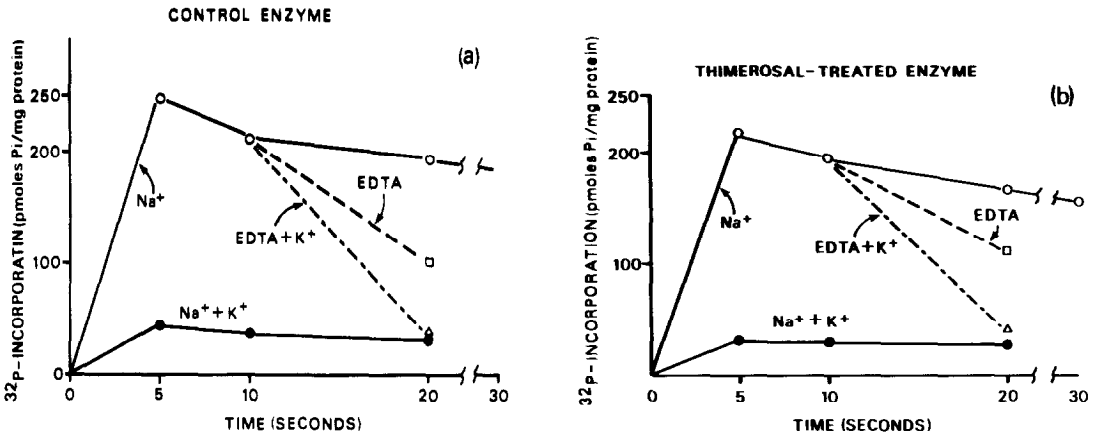

Fig. 2.(a and b). Effects of Na^+ and K^+ on phosphoenzyme formation and breakdown in the control and the thimerosal-treated enzymes. The reactions were done in the presence of (1) Na^++K^+; (2) Na^+ alone; (3) Na^+ with the addition of EDTA after ten seconds; and (4) Na^+ with the addition of EDTA and K^+ after ten seconds. The final concentrations of the indicated reagents were: Na^+, 100 mM; K^+, 20 mM; EDTA, 15 mM. Other conditions and procedures were the same as described before (9).

cast doubts on its validity. The data of these investigators, in contrast to those of Neufeld and Levy (10), have suggested that the same phosphoenzyme is involved as an intermediate in the Na^++K^+-dependent hydrolysis of ATP and the Na^+-dependent hydrolysis of ATP and UTP. If the catalytic center for the formation of phosphoenzyme intermediate is the same for both Na^++K^+-dependent activity and Na^+-dependent activity, one might have expected the selective inhibition of the former activity by thimerosal to be due to the blockade of the K^+ effect on the discharge of the phosphoenzyme. As the data of Fig. 2 show, this is not the case. The phosphoenzyme of the thimerosal-treated enzyme is fully responsive to K^+. Why then is the Na^++K^+-dependent activity inhibited by thimerosal? A plausible explanation is as follows: The findings of Siegel and Goodwin (8), and Post et al. (7) indicate that when K^+ combines with the phosphoenzyme to discharge it, a relatively stable K^+-dephosphoenzyme is formed; and if the enzyme is to recycle, this complex must dissociate. As suggested by Post et al. (7), with the native enzyme in the presence of high ATP concentrations, the dissociation of K^+-dephosphoenzyme is accelerated due

to the binding of ATP to a low affinity site that is distinct from the high affinity catalytic site. Thus, it is sufficient to assume that in the thimerosal-treated enzyme ATP can no longer bind to this low affinity activating site. In this case when Na^+ and K^+ are added in sequence the enzyme is phosphorylated and dephosphorylated in a "normal" way, but only once!

It is apparent from the above that the properties of the thimerosal-treated enzyme as reported here can best be explained by assuming (a) the existence of two distinct nucleotide binding sites on the enzyme; and (b) the selective blockade of one of these sites by thimerosal. A decision on the precise function of the blocked site can not be made on the basis of our data alone.

We should also note that the presence of two ATP binding sites on the enzyme is assumed in a variety of other proposed models for the transport enzyme (11-15). While the discussion of the present findings in the context of each model is premature, we feel that further studies on the properties of the modified enzyme in which one ATP site has been functionally blocked should be of help in the evaluation of the various hypotheses.

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