THE NATURE OF THE TRANSPORT ATPase-DIGITALIS COMPLEX. I. FORMATION AND REVERSIBILITY IN THE PRESENCE AND ABSENCE OF A PHOSPHORYLATED ENZYME[1]

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SUMMARY - The stability of the H^3-cardiac glycoside-Na^+,K^+-ATPase complex was studied at various temperatures. Complex I, formed in the presence of [ATP + Mg^{++} + Na^+], was much less stable than complex II, synthesized in the presence of [Mg^{++} + Pi] or [Mg^{++} + acetate]. Dissociation of the former was prevented by K^+; the more stable complex II was unaffected by K^+.

INTRODUCTION - The Na^+,K^+-ATPase enzyme reaction sequence presumably involves a phosphorylation and a dephosphorylation step(s), both of which are specifically inhibited by cardiac glycosides (1). A tenacious and relatively irreversible binding of radioactive cardiac glycosides to isolated Na^+,K^+-ATPase preparations under certain specific conditions has been described (2-6). Furthermore, data indicate that at least two ionic conditions can stimulate maximal ouabain binding to the enzyme (7,8): (1.) ATP + Mg^{++} + Na^+; (2.) Mg^{++} + Pi. It has been suggested from this that "phosphorylated intermediates" formed under these two conditions are chromatographically identical (9). However, the data in this report do not support this contention since the enzyme-glycoside complex appears to be reversible to varying degrees, depending upon the conditions of formation and the ionic milieu. Furthermore, digitalis binds specifically to either a phospho or dephospho enzyme, suggesting that a "phosphorylated intermediate" is not necessary for the action of cardiac glycosides.

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METHODS -

Enzyme Isolation. Isolation of the Na^+,K^+-ATPase preparation from calf brain was by previously published procedures (10). The enzyme was 95-99% ouabain-sensitive.

Binding and Dissociation Studies. Three mg of enzyme protein were incubated in a final volume of 20 ml, in a small flask containing 10^{-6} M H^3-ouabain, in a medium consisting of 50 mM Tris HCl, pH 7.4, and 1 mM Tris-EDTA at 37°C. Prior additions were made to the flasks as indicated, i.e., 1.25 mM Na_2ATP, 1.25 mM $MgCl_2$, 50 mM NaCl, or 1.25 mM $MgCl_2$ + 1.25 mM Tris PO_4 or 1.25 mM $MgCl_2$ + 1.25 mM Tris-acetate. In each case the flasks were preincubated for 5 minutes at 37°C, and the reaction started by the addition of enzyme. After incubation to achieve optimal binding (5 minutes for Mg^{++} + ATP + Na^+, and Mg^{++} + Pi; 15 minutes for Mg^{++} + OAc), the reaction was terminated by centrifugation at 100,000 x g for 15 minutes at 2-5°C. The pellets were resuspended in 10 ml of 1 mM Tris-EDTA at 0°C; 1 ml of the suspension (containing 300 μg protein) was added to a polycarbonate tube which contained 1 ml of 1 mM Tris-EDTA, with or without K^+. The dissociation reaction proceeded for the indicated times (see Table) and was terminated by centrifugation at 100,000 x g. The pellets were dissolved in 0.3 ml of 0.2 N NaOH and suspended in a scintillation medium of 10% BBS-3 (Beckman Instruments), Fluoralloy (Beckman Instruments) in a toluene base. Radioactivity was counted in a Beckman LS200B Liquid Scintillation Spectrometer, with internal standardization and appropriate H^3-ouabain or H^3-digoxin standards.

RESULTS AND DISCUSSION - Specific maximal binding of H^3-ouabain to the enzyme occurs in the presence of different ligands and appears to be similar in all cases studied (Table 1). Notable

TABLE 1. REVERSIBILITY OF H^3-OUABAIN-Na^+,K^+-ATPase INTERACTION

a) Dissociation of complex at 37° C

Formation Conditions	ATP + Mg^{++} + Na^+		Mg^{++} + Pi		Mg^{++} + OAc	
"Reversibility" Time (Min)	0 K^+	10mM K^+	0 K^+	10mM K^+	0 K^+	10mM K^+
0	100	100	100	100	100	100
5	52	98	98	96	89	86
10	30	92	90	92	84	80
15	28	88	85	86	73	70
20	18	85	78	80	63	66

b) Dissociation of complex at 45° C

Formation Conditions	ATP + Mg^{++} + Na^+		Mg^{++} + Pi		Mg^{++} + OAc	
"Reversibility" Time (Min)	0 K^+	10mM K^+	0 K^+	10mM K^+	0 K^+	10mM K^+
0	100	100	100	100	100	100
5	15	80	72	76	76	82
10	4	64	52	61	63	63
15	3	55	40	50	49	52
20	2	43	38	44	32	42

Tritiated-ouabain binding to Na^+,K^+-ATPase under 3 different conditions was carried out at 37° C. Maximal binding obtained was [ATP + Mg^{++} + Na^+] = 72.3 pmoles H^3-ouabain/mg protein; [Mg^{++} + Pi] = 82.4 pmoles H^3-ouabain/mg protein; [Mg^{++} + OAc] = 23.6 pmoles H^3-ouabain/mg protein. After binding, the complex was separated from the medium (see Methods) and reversibility studies were carried out in 1 mM Tris-EDTA pH 7.4, at 37° C or 45° C, in the presence and absence of 10 mM K^+. Dissociation values are expressed as percent of maximal binding, indicated as 100% (time zero). Pi = inorganic phosphate, tris salt; OAc = acetate, tris salt.

is the binding of ouabain in the presence of Mg^{++} and acetate alone, conditions that make the presence of any phosphorylated intermediate or state highly unlikely. These complexes, however, display different dissociation characteristics depending upon the original ionic environment present during formation. For convenience, we refer to the complex formed in the presence of ATP + Mg^{++} + Na^+ as "unstable" (I), and in the presence of Mg^{++} +

Pi or Mg^{++} + acetate as "stable"(II).

Potassium exerted a stabilizing influence on the "unstable" complex I (Table 1). Sodium also displayed similar activity, but to a lesser extent (11-13). The dissociation (or reversibility) of the complexes was temperature-dependent (Table 1). Although complex II readily dissociated at 45°C, the reversibility of II was much less affected by 10 mM K^+ than was complex I. Similar results were obtained with enzymes from beef heart, dog kidney cortex and medulla, rabbit heart and kidney and rat brain, and when H^3-digoxin was substituted for H^3-ouabain. It should also be emphasized that when the drug was removed from the enzyme, under conditions described, Na^+,K^+-ATPase activity was completely restored (data not shown).

The two ionic conditions, ATP + Mg^{++} + Na^+ and Mg^{++} + Pi, both stimulate maximal binding; it has been suggested that identical phosphorylated intermediates result from the reaction sequence (9). The present data clearly show that the complexes formed are not the same, since their dissociation characteristics are different. Furthermore, potassium is ineffective as a stabilizer of complex II, but effectively stabilizes complex I. The effect of K^+ is partially responsible for results found earlier (4), since we now have data indicating that assay conditions (viz., ATP, Mg^{++}, Na^+ + K^+) also prevent the dissociation of the drug-enzyme complex. Furthermore, these data explain the lack of reversibility recently reported by Yoda and Hokin (14) after binding ouabain to Na^+,K^+-ATPase in the presence of ATP + Mg^{++} + Na^+, and subsequently diluting the complex with the assay medium (containing ATP + Mg^{++} + Na^+ + K^+).

The differences in stabilities of digitalis-enzyme complexes suggest multiple binding conformations of the enzyme protein. It

is clear that a number of different ligands alter the conformation of the enzyme and that digitalis can bind equally well under varying conditions. Furthermore, the digitalis-enzyme interaction is reversible, depending on temperature, ionic ligands and the type of originally formed complex. Phosphorylation of the enzyme is not required for specific formation of the inhibitor-enzyme complex.

These data support the allosteric hypothesis for both pump action and digitalis-induced inhibition of the Na^+,K^+-ATPase.

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