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The Interaction of Ouabagenin and (Na^+ + K^+)-ATPase in the Presence of Na^+, Mg^{2+}, and ATP

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SUMMARY

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(Na^+ + K^+)-ATPase-rich membrane fragments from the electric organ of the electric eel were used to study the association and dissociation of ouabagenin (a reversibly bound cardiac aglycone) in the Na^+-Mg^{2+}-ATP system. To measure these rates, one or three second assays for ATPase were performed using [γ-^{32}P]ATP. This method assays the reversible binding, the binding of the steroid moiety. With this short-period assay, the pseudo-first order association rate constant (K'_a) of 1 μM ouabagenin was found to be 0.78 min^{-1} at 30°—the same as that of 1 μM ouabain. However, the K'_a of 1 μM ouabain was 0.40 min^{-1} by the dilution method which measures the pseudo-irreversible binding, the binding of the sugar moiety. These results support our previous hypothesis that the cardiac steroid binds with the enzyme by the steroid moiety at first, and the pseudo-irreversible binding of sugar moiety follows. In the dissociation of the ouabagenin-(Na^+ + K^+)-ATPase complex, the presence of K^+ in the dissociation medium did not stabilize the complex, unlike the effect of K^+ on the ouabain-enzyme complex. However, by elevating the pH value, the dissociation rate of the ouabagenin-enzyme complex was reduced, like that of the ouabain complex. Moreover, at pH 7.35, ouabagenin dissociates biphasically, and it is concluded that two forms of ouabagenin-(Na^+ + K^+)-ATPase complex exist in the Na^+-Mg^{2+}-ATP system, where the dissociation rate constants (K_d) of these complexes were 0.30 min^{-1} and 2.5 min^{-1} at 30°, respectively. Similar results were observed with the digoxigenin complex, where the K_d values were 0.46 min^{-1} and 3.1 min^{-1} at 30°.

INTRODUCTION

The sodium- and potassium-dependent adenosine triphosphatase [(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). (Na^+ + K^+)-ATPase is specifically inhibited by cardiotonic steroids. Experiments using the radioactive cardiac glycosides, ouabain and digitoxin, have shown that certain ligands are necessary for the binding of (Na^+ + K^+)-ATPase with cardiac glycosides. The two most effective systems are Na^+-Mg^{2+}-ATP and Mg^{2+}-P_i (2). For convenience, we refer to Na^+-Mg^{2+}-ATP as the Type I system and Mg^{2+}-P_i as the Type II system.

Our rate kinetic studies on the interaction between cardiac glycosides and (Na^+ + K^+)-ATPase indicated that cardiac glycosides were bound to the enzyme at the steroid-specific binding site at first, and then this binding resulted in the activation of the sugar-binding site on the enzyme, which led to the binding of the glycoside portion (3, 4). The binding at the sugar site has a high affinity and is nearly irreversible. It may mask the characteristics of the binding at the steroid site, which is the initial step in the cardiac glycoside binding. To elucidate the binding at the steroid site, we must study the cardiac steroid itself, rather than its glycoside. We have already reported on the equilibrium binding of the cardiac aglycone, digoxigenin, to the enzyme in the Type I and Type II systems (5), and on the rate kinetics and the ligand effects on the rate of the cardiac aglycone binding to (Na^+ + K^+)-ATPase in the Type II system (6–8). From these studies, we concluded that the binding of the cardiac aglycone was not an ordinary reversible type of binding and proposed a new reaction sequence containing at least one irreversible step (8).

For the determination of unbound enzyme (enzyme that has remained active), the usual ATPase assay after a 10- to 20-fold dilution to stop the formation of more complex can be used in the case of cardiac glycoside complexes because the cardiac glycoside-enzyme complex is stable enough and no significant dissociation of the complex is observed during the ATPase assay (5, 9).

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However, the cardiac aglycone complex, unlike the glycoside complex, dissociates rapidly upon dilution, and a short-period assay method is needed to measure the association or dissociation rate of the complex. Previously, we used the assay of phosphoprotein from ATP to determine these rates in the Type II complex (6). However, the presence of rather high concentrations (0.1 mM) of ATP as the promoting ligand for Type I cardiac steroid binding makes it difficult to determine the levels of phosphorylated proteins. In this study, these difficulties were overcome by the use of a highly active enzyme preparation and a short-period assay of ATPase using [γ-^{32}P]ATP to measure the remaining or recovered active enzyme. The present study has also focused on comparison between the cardiac aglycone (mainly ouabagenin)-$(Na^+ + K^+)$-ATPase complex and the glycoside (ouabain)-enzyme complex.

MATERIALS AND METHODS

The enzyme used here was the $(Na^+ + K^+)$-ATPase-rich membrane fragments obtained from the electric organ of the electric eel. The specific activity of the preparation obtained was approximately 11 ± 1 μmoles of ADP per milligram per min at 30° and was approximately 15-fold higher than that of the NaSCN-treated beef brain enzyme used previously for the studies of the Mg^{2+}-P_i system. This preparation is as stable as the beef enzyme and does not contain any detectable Na^+-independent ATPase activity. The preparation method will be described elsewhere (9a).

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (10) with the modification of Post and Sen (11). This preparation was purified every week by DEAE-Sephadex chromatography to reduce the inorganic ^{32}P content as follows: The DEAE-Sephadex column (0.7 cm × 4 cm) was equilibrated with 100 mM imidazole-HCl of Tris-HCl buffer, which had a pH value that could vary from 6.5 to 8.5. Less than 4 ml of the sample solution, which should contain less than 100 mM Tris or imidazole buffer, was charged on the column. After washing with 50 ml of the same 100 mM buffer, the concentration of the buffer was increased to 200 mM to elute the purified [γ-^{32}P]ATP. When this chromatography was carried out at 0–4°, the P_i to ATP ratio in the obtained preparation was less than 0.01.

Ouabagenin was prepared by the method of Mannich and Siewert (12) as reported previously (13). For the short-period assay methods described below, we used the semirapid mixing apparatus described previously (14). By using this apparatus, an accuracy of approximately ±5% of the ATPase activity for the 1-sec or 3-sec assay period was obtained.

Association of cardiac aglycones with $(Na^+ + K^+)$-ATPase. A mixture (0.4 ml) containing 40–60 μg of enzyme, 1.0 μmole of Mg^{2+}, 50 μmoles of Na^+, and 10 μmoles of imidazole HCl (pH 7.35) was stirred in a reaction tube at 30°, and the cardiac aglycone association reaction was started by the injection of 0.1 ml of a mixture containing 1 mM Tris-ATP and cardiac aglycone. At various intervals during the association, an assay of the remaining enzyme activity was started by the injection of 0.1 ml of solution containing [γ-^{32}P]ATP (5–10 × 10^6 dpm) and 120 mM K^+. After 1 or 3 sec, the reaction was quenched by the injection of 0.2 ml of 60% perchloric acid containing 0.1 mM unlabeled P_i. After the addition of 0.5 ml of 7% sodium molybdate solution and 2 ml of butyl acetate, the mixture was agitated briefly, and the butyl acetate layer was centrifuged to clarify it using a clinical centrifuge. The radioactivity in the 1 ml of butyl acetate extract was measured by the Cerenkov method after dilution with acetone. The radioactivity in the butyl acetate extract was assayed in quadruplicate at five or six intervals. In the control experiments, the order of addition of the [γ-^{32}P]ATP solution and the perchloric acid solution was exchanged. Under the experimental conditions shown in Figs. 2 and 3, the radioactivity of P_i did not change more than 5% between the 10-sec and 90-sec intervals. From the differences between them, the inhibition curve shown in Fig. 2 was obtained. The slope of the curve was the pseudo-first order rate constant (K'_a) as interpreted for cardiac glycoside (3) or aglycone (6) association in the Type II complex. As shown in Fig. 1, the K'_a value is directly proportional to the concentration of ouabagenin, and we can calculate the second-order association rate constant with the usual methods.

Measurement of the dissociation rate of ouabagenin or digoxigenin enzyme complex. To reach equilibrium, a mixture of the eel enzyme (0.4–1.0 mg/ml), 100 mM NaCl, 2 mM $MgCl_2$, 2.5 mM unlabeled ATP, 4 μM of ouabagenin or digoxigenin, and 25 mM imidazole-HCl buffer (or Tris-HCl buffer) was incubated for 20–30 min at 30°. The dissociation was started by the dilution of the equilibrated mixture (50 μl) with 2 ml of 20 mM imidazole-HCl buffer (or Tris-HCl buffer) and, at the cited intervals, 0.1 ml of the assay mixture containing [γ-^{32}P]ATP, Mg^{2+}, Na^+, and K^+ was injected. The final concentrations in the reaction mixture during this assay period were 0.1 mM ATP (10^6–10^7 dpm), 2 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, and 25 mM buffer. After a 1- or 3-sec incubation, the ATPase reaction was quenched by the addition of 0.2 ml of 60% perchloric acid containing 1 mM unlabeled P_i. The same procedure for the [γ-^{32}P]P_i assay to determine the association rate was then carried out.

In order to calculate the inhibition by the aglycones, control experiments were carried out under the same conditions without any cardiac aglycone. No significant change in enzyme activity was observed during the incubation after dilution (less than 5 min). From such inhibition data at five or six intervals, a stability curve as shown in Figs. 3 and 4 was obtained, and from the slope of the curve the dissociation rate constant (K_d) was calculated.

In some experiments for the association and dissociation rates of ouabain and other cardiac glycosides, the dilution method was used. The details of this method have been reported (3, 4).

RESULTS

Table 1 shows the association and dissociation rate constants (K_a and K_d) of ouabain with the new $(Na^+ + K^+)$-ATPase-rich membrane fragments. In this case, the

TABLE 1

Association and dissociation rate constants of ouabain to (Na^+ + K^+)-ATPase measured by the dilution method

The values were obtained at 30° except as indicated.

	Electric eel enzyme	NaI-treated beef brain enzyme[a]
Second-order association rate constant (K_a) (μM^{-1} min^{-1})		
		1.32
Type I	0.41 ± 0.03	0.64 (25°)
Type II	0.22	0.80 (25°)
Dissociation rate constant (K_d) (hr^{-1})		
Type I	0.46	0.87
Type II	0.095	0.42
K_d/K_a ($\times 10^{-9}$ M)		
Type I	18.7	22.7 (25°)
Type II	7.2	8.8 (25°)

[a] These values were calculated from data reported previously (3, 4, 9).

measurements were carried out by the dilution method (3) in which the remaining ATPase activity was measured after stopping the interaction between ouabain and the enzyme by dilution. As described below, this method can follow the binding of the sugar moiety, which is considered to occcur after the activation of the sugar-specific site on the enzyme by the binding of the steroid (aglycone) moiety of ouabain to the steroid-specific site of the enzyme. As compared with the NaI-treated beef brain microsome preparation, both the K_a and K_d of the eel enzyme were lower, i.e., the association was slower, but the stability of the ouabain complex was greater than that of the beef brain enzyme. However, for the ratio of K_d to K_a, the differences between the beef and eel enzymes were smaller. These relationships were not specific to the case of ouabain, since with the other cardiac glycosides, convallatoxin and digitoxigenin monodigitoxide, similar relationships were observed (data not shown).

The pseudo-first order rate constant of ouabagenin association measured by the short-period assay was directly proportional to the concentration of ouabagenin (Fig. 1); therefore, the association of ouabagenin seemed to follow second-order rate kinetics, and its second-order rate constant was 0.76 min^{-1} μM^{-1} at 30°.

When the association rate of ouabain was measured by this short-period assay, the pseudo-first order association rate constant of 1 μM ouabain was 0.78 min^{-1} at 30°, as

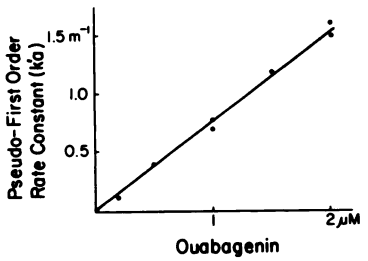

FIG. 1. *Direct proportionality of the pseudo-first order association rate constant to the concentration of ouabagenin*

This experiment was performed at 30°. See details of the procedure in the text.

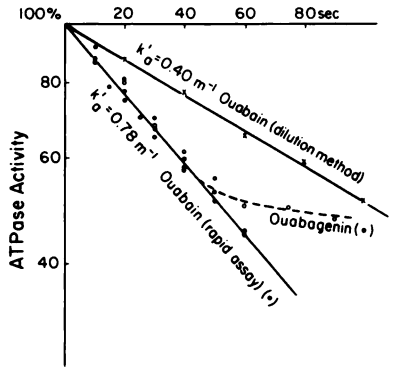

FIG. 2. *Comparison of the association rates of ouabain and ouabagenin in the Na^+-Mg^{2+}-ATP system*

Rapid assay: The enzyme (50 μg/ml) was treated at 30° in a mixture (2 ml) of 100 mM Na^+, 4 mM Mg^{2+}, 2 mM ATP, and 1 μM ouabain (●) or ouabagenin (○). At the indicated intervals, 0.1 ml of the assay medium containing 8×10^{-7} dpm of [γ-^{32}P]ATP and 40 μmoles of KCl were added; after a 1-sec hydrolysis, the liberated inorganic phosphate was assayed as described in the text.

Dilution method: The association conditions with ouabain were the same as above, but at the indicated intervals, 100 μl of the reaction mixture were diluted with 4 ml of buffer, and the ATPase activity in the diluted aliquot was measured.

shown in Fig. 2. This value is coincident with the value of ouabagenin and is different from the association rate of ouabain measured by the dilution method, which has a pseudo-first order rate constant of 0.40 min^{-1}.

As expected from the K_d values of the Type II cardiac aglycone complexes reported previously (6), the K_d values of Type I ouabagenin (aglycone) complex were more than 100-fold greater than those of the ouabain (glycoside) complex (Fig. 3). This difference apparently originated from the specific binding of the sugar moiety of the cardiac glycoside as predicted previously (3, 4, 9).

It has been observed that the dissociation rate of the Type I complexes of cardiac glycosides and (Na^+ + K^+)-ATPase from dog heart, rat brain, or beef brain enzyme are reduced by the presence of K^+ in the dissociation medium (15–17). As shown in the *inset* of Fig. 3, a similar effect of K^+ on the dissociation of ouabain was also observed in the eel enzyme. However, the dissociation rate constants of ouabagenin were 1.28 min^{-1} (30°) or 0.40 min^{-1} (20°) in the absence of K^+, and 1.24 min^{-1} (30°) or 0.36 min^{-1} (20°) in the presence of 2 mM K^+ (Fig. 3). In another set of experiments using different concentrations of K^+ in the dissociation medium, the dissociation rate constants of ouabagenin at 30° were 1.20 min^{-1} without K^+, 1.22 min^{-1} with 5 mM K^+, and 1.19 min^{-1} with 10 mM K^+. In conclusion, the effect of K^+ on the dissociation of a cardiac glycoside is apparently specific to the binding of the sugar moiety.

It is also another characteristic of the Type I glycoside complex that the dissociation rate of the complex is reduced with an increase of pH in the dilution medium (7). In the case of cardiac aglycone complexes, a similar effect of pH on the dissociation rate was observed in the case of ouabagenin and digoxigenin, as shown in Figs. 4 and 5.

Moreover, a biphasic dissociation of Type I ouabagenin or digoxigenin complexes was observed at some values of

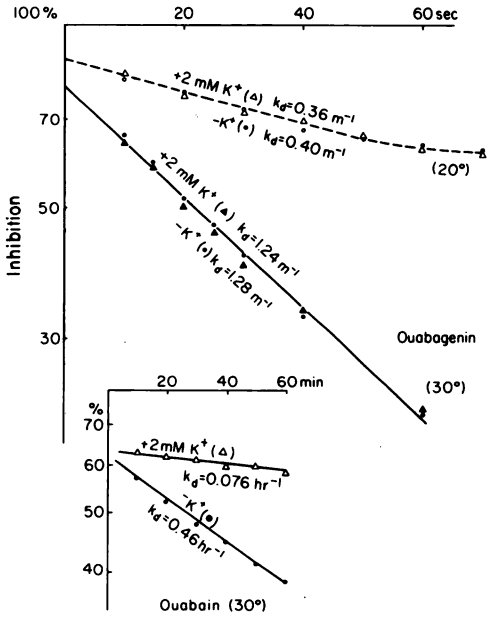

FIG. 3. *Effect of K^+ on the dissociation of ouabagenin $(Na^+ + K^+)$-ATPase complex*

A mixture of the enzyme (0.5 mg/ml), 4 μM ouabagenin, 100 mM Na^+, 2.5 mM Mg^{2+}-ATP, and 25 mM imidazole-HCl buffer (pH 7.35) was incubated for 20–30 min at 20° (○, △) or 30° (●, ▲). The dissociation was started by the 40-fold dilution of the equilibrated enzyme mixture (50 μl) with the same imidazole buffer with (▲, △) or without (●, ○) 2 mM KCl. At the indicated intervals, the assay medium containing [γ-^{32}P]ATP was added. After 1 sec, the hydrolysis was terminated with perchloric acid, and the liberated inorganic phosphate was assayed as described in the text. The concentrations of the ligands during the ATPase assay were 0.5 mM ATP, 1.5 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, and 50 mM imidazole-HCl buffer (pH 7.35).

Inset, the K^+ effect on the dissociation of ouabain complex. The conditions of phosphorylation and dissociation procedures were the same as those of ouabagenin except for the replacement of 1 μM ouabain for 4 μM of ouabagenin and the 10-min incubation of inhibition. For the assay of the recovered enzyme, a suitable aliquot of the diluted solution was analyzed by the linked pyruvate kinase-lactate dehydrogenase spectrophotometric method. The dissociation curve in the absence of K^+ is shown by ●——●, and that in the presence of 2 mM K^+ is shown by △——△.

pH. From the assumption that the first rapid phase contains a rapid dissociation complex and the slow dissociation complex whereas the second slow phase contains only a slow dissociation complex, the K_d values of the slow and rapid dissociation complexes could be calculated. These results are shown in Table 2. From Table 2 we may conclude that two different complex forms of cardiac steroid and $(Na^+ + K^+)$-ATPase are present, dissociate independently, and that their ratio varies with pH.

DISCUSSION

Since 1973, we have presented evidence from the structure-activity relationships of the association and dissociation rate constants (3, 4) that the glycoside moiety of the cardiac glycoside can bind with a sugar-specific site on $(Na^+ + K^+)$-ATPase through the interaction of the 3′hydroxyl and other functional groups. Recently, Fullerton *et al.* (18) presented more support for this sugar-

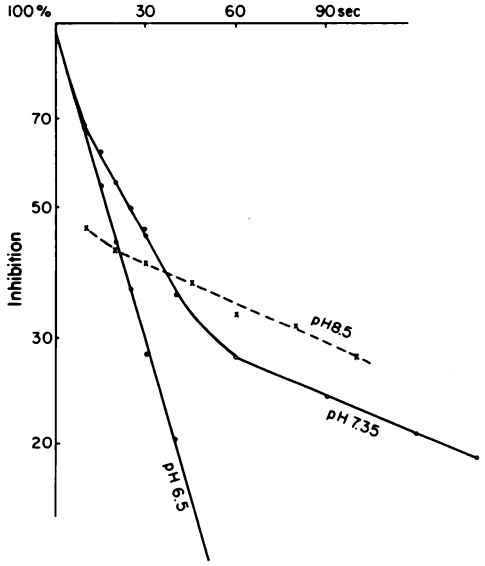

FIG. 4. *Stability of Type I ouabagenin complex at 30°*

The experimental conditions for the equilibrated ouabagenin-enzyme mixture were the same as in Fig. 3. The equilibrated mixture (50 μl) was diluted with 2 ml of 20 mM imidazole buffer (or Tris-HCl buffer at pH 8.5), and, at the intervals cited, [γ-^{32}P]ATP and salt mixture were added for ATPase activity. The final concentrations of ligand during the ATPase assay were the same as those in Fig. 3. After a 3-sec incubation, the reaction was terminated by excess perchloric acid, and the liberated $^{32}P_i$ was measured.

binding hypothesis from a molecular analysis of a cardiac glycoside. The binding of the glycoside moiety is very stable and may mask the nature of the interaction between the cardiac aglycone and the steroid-specific site on the enzyme which is believed to be the initial step in the cardiac glycoside binding. Although no data except our previously reported results have been published about the association and dissociation rates of Type I

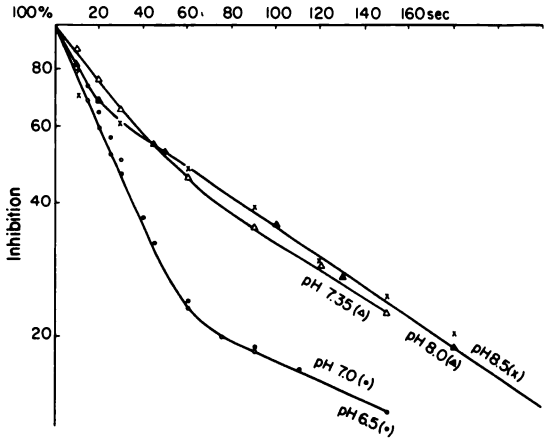

FIG. 5. *Stability of Type I digoxigenin complex at 30°*

A mixture of the eel enzyme (0.84 mg/ml), 100 mM Na^+, 2.5 mM ATP, 4 mM Mg^{2+}, 1 mM EDTA, 25 mM buffer, and 4 μM digoxigenin was incubated for 20–30 min. The equilibrated mixture (50 μl) was diluted with 2 ml of 25 mM buffer (bis-Tris buffer at pH 6.5, imidazole-HCl buffer at pH 7.0 and 7.35, and Tris-HCl buffer at pH 8.0 and 8.5); at the intervals shown, [γ-^{32}P]ATP and salt mixture were added for the ATPase assay. After a 1-sec incubation, the liberated $^{32}P_i$ was measured. The assay conditions were the same as in Fig. 3.

TABLE 2

Dissociation rate constants of ouabagenin and digoxigenin at various pH levels

These values are calculated from the slopes shown in Figs. 4 and 5, based on the assumption that the initial rapid phase is the sum of a rapid dissociation and a slow dissociation, which is equal to the slow phase shown in Figs. 4 and 5.

	pH				
	6.5	7.0	7.35	8.0	8.5
			min^{-1}		
Ouabagenin					
Initial phase	2.5		1.2		
Slow phase			0.30		0.30
Calculated rapid phase			2.7		
Digoxigenin					
Initial phase	1.68	1.5	0.83	?	
Slow phase	0.43	0.49	0.46	0.47	0.46
Calculated rapid phase	3.2	2.9	3.1		

and Type II cardiac aglycone and $(Na^+ + K^+)$-ATPase, it has been suggested that both rates, especially the dissociation rate, are higher than those of cardiac glycosides because of the lack of sugar moiety in the cardiac aglycone. The present results show that the dissociation rate of ouabagenin is approximately 150-fold greater than that of ouabain at 30° in the Type I complex. This difference seems to be greater than that of the Type II complex previously reported (81-fold greater) (6), even though the source of the enzyme was different.

When this short-period assay method was used for the ouabain association, the K_a value obtained was higher than that obtained by the dilution method. The dilution method can estimate only the rate of the pseudo-irreversible binding, namely, the binding by the sugar moiety, but the short-period assay method can determine the rate of the reversible binding of the steroid moiety. The interesting point is that the K_a value of ouabain obtained by the short-period assay is the same as that of ouabagenin, the steroid moiety of ouabain. This correspondence supports our previous suggestion from the structure activity relationship studies that the binding of the steroid portion is coincident with or prior to the rate-limiting step of the association of cardiac glycoside. This also supports our model of the binding mechanism, where the steroid moiety of the cardiac glycoside reversibly binds to the steroid-specific site on the enzyme at first, which results in the activation of the sugar-binding site, with the consequent binding of the glycoside portion (3, 4).

The effect of K^+ on the dissociation of Type I ouabain-enzyme complex is well known, but such an effect is not observed in the ouabagenin complex (Fig. 3). Therefore, this K^+ effect is specific to the sugar-binding site. We have already shown that this K^+ effect is observed in the Type I binding of the first and second sugar moieties and not in the third and fourth sugar moieties of the cardiac oligodigitoxides (19, 20). From these results, the K^+ effect on the cardiac glycoside-enzyme complex seems to be limited to a narrow region on the enzyme related to the first and second sugar sites.

The dissociation rate of the type I ouabain complex varies with pH (7, 21). This is another notable difference from the Type II complex. This pH effect seems to be dependent on the steroid-binding site because this effect was observed in the complexes of ouabagenin and digoxigenin (Figs. 4 and 5). In the previous study of the ouabain complex (7), the coexistence of two forms of ouabain-enzyme complex was not indicated in the Type I complex, but in the case of cardiac aglycone complexes, biphasic dissociations were observed. Assuming the coexistence of the rapid dissociation form and the slow dissociation one in the cardiac aglycone-enzyme complex and their independent dissociation, the dissociation rate constant of each form is calculated at various pH values (Table 2). Each value does not vary with pH, but the relative content ratio of each form varies with pH. Therefore, we may conclude that two forms of Type I cardiac aglycone-$(Na^+ + K^+)$-ATPase complex exist, and this conclusion may explain the pH dependency of the dissociation rate on the Type I cardiac glycoside-$(Na^+ + K^+)$-ATPase complex. At the moment, we cannot interpret the differences between these two forms, but it is notable that the dissociation rate constant of the rapid-dissociation form is approximately 8-fold greater than that of the slow-dissociation rate constant in the case of ouabagenin or digoxigenin.

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