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Structure-Activity Relationships of Cardiotonic Steroids for the Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase

III. Dissociation Rate Constants of Various Enzyme–Cardiac Glycoside Complexes Formed in the Presence of Sodium, Magnesium, and Adenosine Triphosphate

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SUMMARY

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The dissociation rate constants (k_d) of cardiac monoglycoside–($Na^+ + K^+$)-ATPase complexes formed in the Na^+-Mg^{2+}-ATP system (type I complex) were determined by enzymatic assay after dilution. The k_d value of each cardiac glycoside–enzyme complex thus formed was greater than that of the complex formed in the Mg^{2+}-P_i system (type II complex). Whereas the k_d values of type II complexes were dependent only on the sugar moiety, the k_d values of type I complexes were affected by both the steroid and sugar moieties. For cardiac glycoside–enzyme complexes in which the sugar moiety was the same, the stability of type I complexes increased in the order: digitoxigenin glycoside < strophanthidin glycoside < digoxigenin glycoside and strophanthidin glycoside < ouabagenin glycoside. If the steroid moiety was the same, the stability increased in the order: digitoxide ≒ 6-deoxyglucoside ≒ fucoside < 6-deoxyguloside < rhamnoside. Methylation of the sugar 3′-hydroxyl group decreased stability. These data indicate that in type I complexes a sugar-specific site(s) on the enzyme binds the sugar moiety at the 2′-α- and 3′-α or β-hydroxyl groups by hydrogen bond(s), and at the 5′-α-methyl group by a hydrophobic bond. The activation energy of this dissociation was approximately 30 kcal/mol with all the cardiac monoglycosides tested. The differences in k_d values between type I and type II complexes indicate that the two are distinct, possibly as a result of conformational differences in the sugar-specific site of the ($Na^+ + K^+$)-ATPase.

INTRODUCTION

Inhibition of ($Na^+ + K^+$)-ATPase by cardiotonic steroids has been studied in many laboratories. Experiments using radioactive ouabain and digoxin (1–4) have established that a cardiac glycoside–enzyme complex is formed in the presence of certain

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ligands. The two most effective ligand systems are the Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP systems (2). Inhibition of $(Na^+ + K^+)$-ATPase by cardiac glycosides does not occur under equilibrium or steady-state condition (5), and therefore we wished to re-examine the structure-activity relationships of cardiac glycoside inhibition by measuring association rate constants and dissociation rate constants in the presence of suitable ligands. Several similar studies have considered dissociation of the ouabain-enzyme complex (6–10).

In a previous paper (11) the effects of cardiac monoglycoside structure on the association rate constants of complexes formed in both the Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP systems were reported. The association rate constant was solely dependent on the nature of the steroid moiety of the cardiac glycoside in either system. The dissociation rate constants of various cardiac glycoside–enzyme complexes formed in the presence of Mg^{2+} and P_i were dependent on the nature of the sugar moiety of the cardiac glycoside, and evidence was presented that the 3′-hydroxyl and 5′-α-methyl groups were bound to a sugar-specific site on the enzyme (5).

This paper reports the dissociation rate constants of various cardiac monoglycoside–$(Na^+ + K^+)$-ATPase complexes formed in the Na^+-Mg^{2+}-ATP system. For convenience we refer to the cardiac glycoside–$(Na^+ + K^+)$-ATPase complex formed in the Na^+-Mg^{2+}-ATP system as a type I complex, and to that formed in the Mg^{2+}-P_i system as a type II complex. This notation is the same as that of Van Winkle *et al.* (9), who designated similar ouabain-enzyme complexes as complexes I and II.

MATERIALS AND METHODS

All cardiac glycosides, the enzyme preparation (NaI-treated microsomes from beef brain), and the assay procedure used in this study were the same as reported previously (5).

The method for determining the dissociation rate constants was essentially the same as used previously to determine the k_d values of type II complexes, except for the procedure for forming the complex. Between 0.1 and 0.6 mg of enzyme preparation was incubated in a final volume of 0.25 ml containing 0.1–0.6 μM cardiac glycoside, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, and 20 mM imidazole HCl buffer, pH 7.3. After 2–10 min of incubation, the interaction between glycoside and enzyme was stopped by dilution with 5.0 ml of 1 mM Tris-EDTA (pH 7.3), and the incubation was continued in order to measure the dissociation as described previously (5).

The dissociation rate constants (k_d) were calculated exactly as described for the type II complexes reported previously (5).

RESULTS

Difference between k_d values of type I and II complexes. The recovery of enzyme activity from both type I and II complexes followed first-order kinetics (Fig. 1). The slopes of these curves represent the dissociation rate constants for the designated cardiac glycoside–enzyme complexes. It can be seen from Fig. 1 and Table 1 that for all 11 cardiac glycosides included in this study, the k_d value of the type I complex for a given glycoside is greater than that of the type II complex.

Effect of aglycone moieties on k_d as a function of temperature. As reported previously (5), the k_d values of type II complexes were solely dependent on the nature of the sugar moiety and were not affected by alteration of the steroid moiety (aglycone). In the case of type I complexes, however, the steroid moiety as well as the sugar moiety influenced the k_d value (Figs. 2 and 3). The results obtained with two rhamnosides (Fig. 2) and three digitoxides (Fig. 3) show that stability increased in the same order as the I_{50} values[1] or the number of hydroxyl groups in the steroid moiety in complexes containing the same sugar moiety.

Effect of sugar moiety on k_d for type I complexes as a function of temperature. Digiproside is the 4′-epimeride of digitoxigenin 6-deoxyglucoside, and the k_d values of these two glycosides were almost the same (Fig. 4). Therefore a configurational change in the 4′-

[1] Since cardiac steroids are reversible inhibitors (12), the I_{50} value is the K_i value in the assay system.

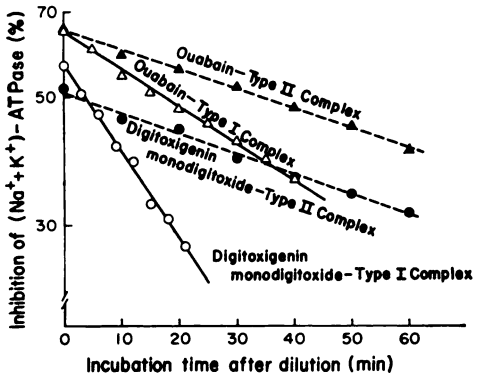


FIG. 1. *Stability of type I and type II complexes after dilution*

In the case of the type I complex, enzyme, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, and 0.2 μM cardiac glycoside were incubated for 7–10 min at 30° for ouabain or 25° for digitoxigenin monodigitoxide. The reaction was stopped by 20-fold dilution with 1 mM Tris-EDTA solution, and enzyme inhibition was determined at suitable intervals. In the case of the type II complex, 1 mM $MgCl_2$ and 1 mM Tris-phosphate were used instead of NaCl, $MgCl_2$, and ATP, and incubation time was 5–7 min. Other conditions were the same as described for the type I complex.

TABLE 1

Dissociation rate constants of various cardiac monoglycoside-$(Na^+ + K^+)$-ATPase complexes at 25°

Glycoside	k_d of type I complex	k_d of type II complex[a]
	hr^{-1}	hr^{-1}
Ouabain	0.35	0.22
Convallatoxin	0.45	0.23
Deglucocheirotoxin	0.71	0.39
Digoxigenin monodigitoxide	0.72	0.42
Helveticoside	0.98	0.48
Digitoxigenin monodigitoxide	1.90	0.44
Cymarin	1.69	0.72
Digiproside	1.77	0.76
Digitoxigenin 6-deoxyglucoside	1.71	0.80
Odoroside H	3.01	1.28
Digitoxigenin-3-tetrahydropyranyl ether	3.36	1.24

[a] Calculated from data reported previously (5).

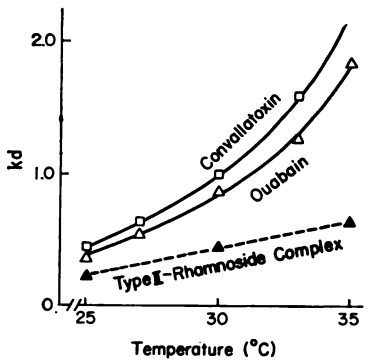
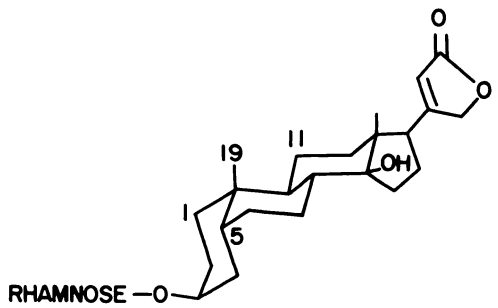


FIG. 2. *Dissociation rate constants (k_d) of type I rhamnoside-enzyme complexes at various temperatures*



The structure of each compound is shown in the following:

Compound	Aglycone	Substitution
Convallatoxin (□)	Strophanthidin	19-CHO, 5-β-OH
Ouabain (△)	Ouabagenin	19-CH_2OH, 1-β-OH 5-β-OH, 11-α-OH

hydroxyl group probably does not affect k_d.

Although deglucocheirotoxin and convallatoxin are 5′-epimers, the change in the 5′-methyl group from α (D series) to β (L series) increased the k_d value (Fig. 5).

In contrast with type II complexes, in which the k_d of deglucocheirotoxin differed only slightly from the k_d of helveticoside, the k_d of the latter was larger than that of the former in the type I complex (Fig. 5). In these glycosides the 4′-hydroxyl groups are in opposite configuration, and there is an additional 2′-α-hydroxyl in deglucocheirotoxin. Since the configuration of the 4′-hy-

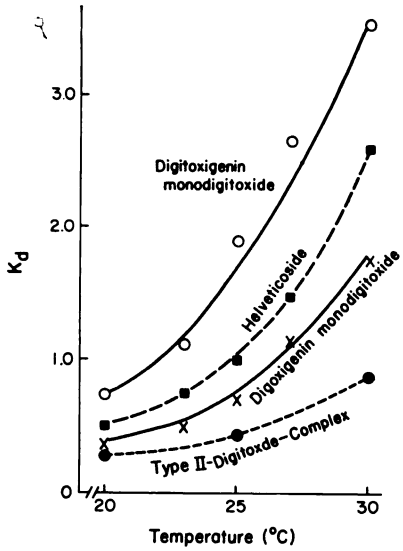
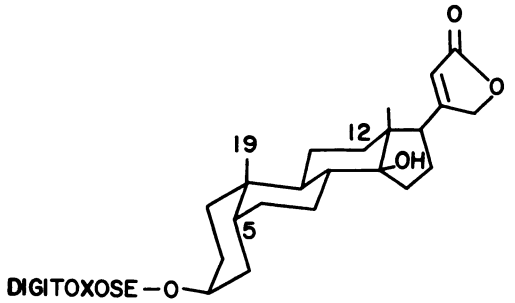


FIG. 3. *Dissociation rate constants (k_d) of type I digitoxide-enzyme complexes at various temperatures*



The structure of each compound is shown in the following:

Compound	Aglycone	Substitution
Digitoxigenin monodigitoxide (○)	Digitoxigenin	19-CH_3
Helveticoside (■)	Strophanthidin	19-CHO, 5-β-OH
Digoxigenin monodigitoxide (×)	Digoxigenin	19-CH_3, 12-β-OH

droxyl group does not appear to affect k_d, as reported above, the decrease in k_d in deglucocheirotoxin may have been due to the 2′-α-hydroxyl group.

Digitoxigenin monodigitoxide had almost the same k_d as digitoxigenin 6-deoxyglucoside (Fig. 4). The structural differences between these two compounds are the configurational change of the 3′-hydroxyl group and an additional 2′-α-hydroxyl group in digitoxigenin 6-deoxyglucoside. Since the addition of the 2′-α-hydroxyl group seemed to increase the stability of type I complexes, changing the 3′-hydroxyl group from α(digitoxide) to β (6-deoxyglucoside) presumably decreases stability.

As shown in the case of cymarin vs. helveticoside (Fig. 5) and odoroside H vs. digiproside (Fig. 4), changing the 3′-hydroxyl group to methoxyl increased the k_d value. The 3-tetrahydropyranyl ether of digitoxigenin, which is considered to be the 2,3,4-trideoxypyranoside (no hydroxyl groups in the sugar), had a higher k_d than odoroside H (Fig. 4). This difference might be due to the 2′-α-hydroxyl group of odoroside H, as described above.

Arrhenius activation energy of dissociation of type I complex. The Arrhenius activation energy of the dissociation of each type I complex formed by 11 cardiac glycosides was calculated using the data shown in Figs. 2–5. These results are shown in Table 2, in which all values are around 30 kcal/mole.

DISCUSSION

The present results concerning the k_d values of type I complexes indicate significant quantitative and qualitative differences from those of type II complexes, although only some quantitative differences were found in the association rate constants between type I and II complexes (11).

Quantitatively, type I is less stable than type II, as shown in the 11 different cardiac glycoside–enzyme complexes, in agreement with previously reported observations on the ouabain-enzyme complex (6, 7). Qualitatively, there are two salient differences: the effects of the aglycone moiety on k_d, and the influence of the functional groups of the sugar moiety on binding to the enzyme.

In the two sets of various cardiac glycosides having a common sugar moiety (rhamnosides and digitoxides), the k_d values of type I complexes were influenced by the nature of the steroid moiety. The reason for this is not obvious, but we offer a tentative explanation. After dilution the dissociation can be summarized as follows:

Dissociation of the sugar moiety from the

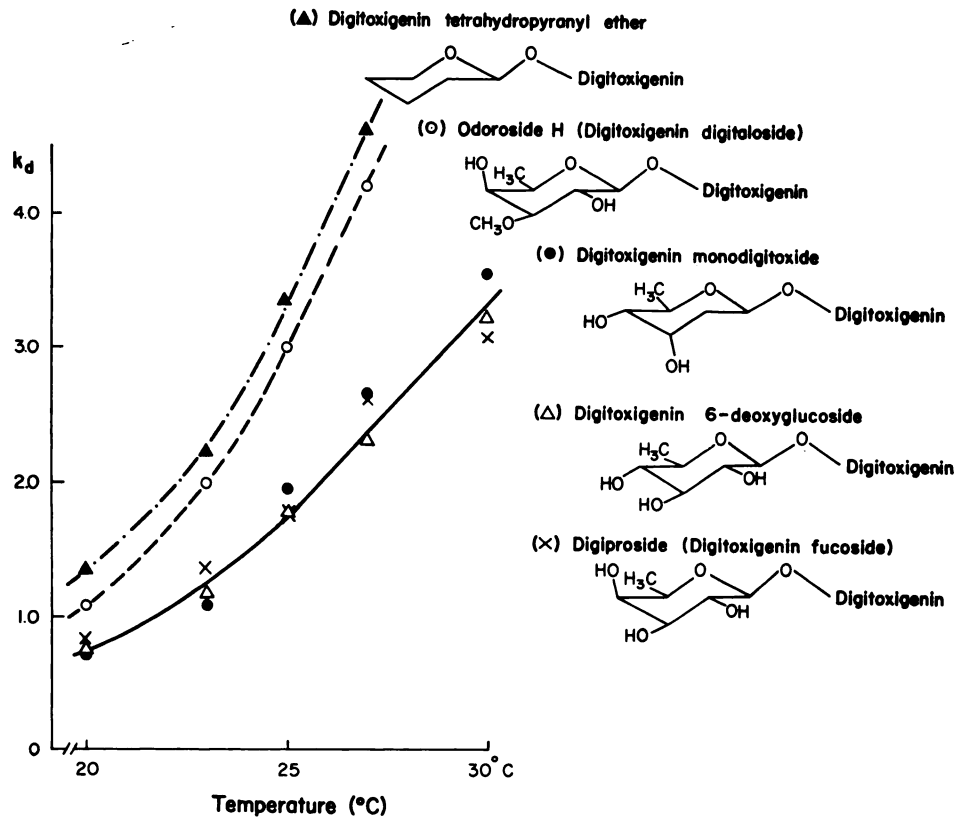


FIG. 4. *Dissociation rate constants (k_d) of type I digitoxigenin glycoside-enzyme complexes at various temperatures*

sugar-specific site:

$$EI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A \qquad (1)$$

Rearrangement of the sugar-specific site from the active to the inactive form:

$$A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} B \qquad (2)$$

Dissociation of the steroid moiety from the steroid-specific site:

$$B \overset{k_3}{\longrightarrow} E + I \qquad (3)$$

If k_3 is much greater than k_2 and k_{-2}, a steady state is established in step 1, and step 2 is the rate-determining step. This will be given by

$$\frac{d[E]}{dt} = -\frac{d[EI]}{dt} = k_2[A] = \frac{k_1 k_{-2}}{k_{-1} + k_2}[EI] \qquad (a)$$

$$k_d = \frac{k_1 k_2}{k_{-1} + k_2}$$

If, however, both reaction rates in steps 1 and 2 are increased—that is, if k_3 is not much greater than k_2 or k_{-2}—then steady states may be established in both steps 1 and 2. Therefore

$$[B] = \frac{k_1 k_2}{k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3}[EI]$$

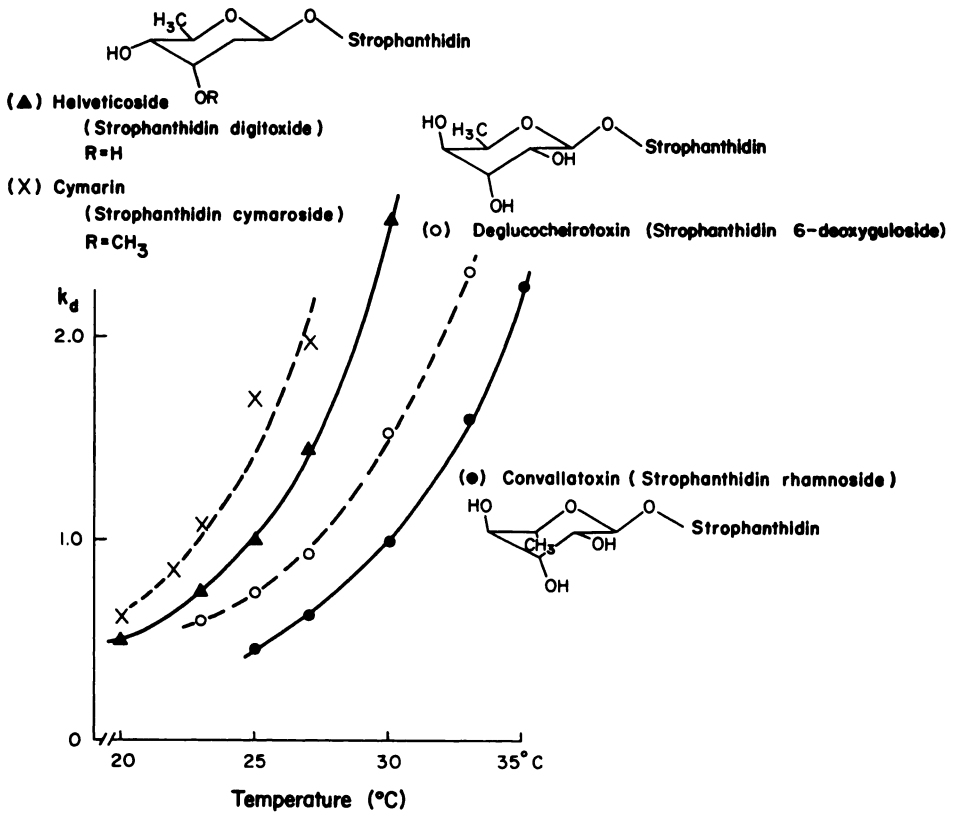


FIG. 5. *Dissociation rate constants (k_d) of type I strophanthidin glycoside–enzyme complexes at various temperatures*

and the whole reaction rate is

$$\frac{d[E]}{dt} = k_3[B]$$

$$= \frac{k_1k_2k_3}{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}[EI] \quad \text{(b)}$$

$$k_d = \frac{k_1k_2}{(k_{-1}k_{-2}/k_3) + k_{-1} + k_2}$$

Therefore the whole reaction rate follows not Eq. (a) but Eq. (b); that is, the reaction rate of step 3 influences k_d.

The above kinetic explanation suggests that the differences in k_d values among the various cardiac glycoside–enzyme complexes containing the same sugar moiety but different steroid moieties might be the result of a decrease in the dissociation rate constant in step 3, or of an increased rate in step 2. An increase in the number of hydroxyl groups in the steroid moiety also might reduce the dissociation rate of the steroid moiety from the steroid-specific site of the type I complex. This tentative conclusion should be tested by other methods; for example, by measuring the k_d values of complexes between the respective aglycones and the enzyme.

The influence of the steroid moieties of the cardiac glycosides on k_d values of type I complexes has made studies on the structure-activity relationships of type I complexes more difficult than on those of type II complexes. Nevertheless, comparison of k_d values for glycosides containing the same aglycone may give some information concerning the structure-activity relationships of the sugar moieties. It is assumed that the binding between the sugar moiety of the cardiac glycoside and the sugar-specific site on the enzyme follows the same structure-activity relationship in both types of complexes, even though

TABLE 2

Activation energy of dissociation of cardiac monoglycoside-($Na^+ + K^+$)-ATPase complexes

Glycoside	Activation energy	
	Type I complex	Type II complex[a]
	kcal/mole	
Ouabain	29	22
Convallatoxin	29	22
Deglucocheirotoxin	28	22
Digoxigenin monodigitoxide	31	20
Helveticoside	30	20
Digitoxigenin monodigitoxide	30	20
Digitoxigenin 6-deoxyglucoside	30	22
Digiproside	28	22
Cymarin	33	22
Odoroside H	35	17
Digitoxigenin-3-tetrahydropyranyl ether	32	18

[a] Data previously reported (5).

the steroid moieties of the glycoside in these complexes are not the same. A comparison of k_d values for various strophanthidin- and digitoxigenin-glycosides indicates that the 3′-hydroxyl and 5′-α-methyl groups (which had been suggested to bind to the sugar-specific site of the enzyme in type II complexes) also stabilize the type I complex, and that the 3′-α-hydroxyl group has a greater affinity than the 3′-β-hydroxyl group. An additional feature of the type I complexes is that the 2′-α-hydroxyl group binds to the enzyme. The binding between the 3′-hydroxyl group and the enzyme in type I complexes involves a hydrogen bond, as in type II, since type I complexes with 3′-methoxylglycosides (cymarin and odoroside H) have less stability than those with 3′-hydroxyl groups. The 2′-α-hydroxyl and 5′-α-methyl groups of the sugar moiety might bind to the enzyme by hydrogen bonding and hydrophobic bonding, respectively, but the evidence for this is weak at the moment.

Since for a given cardiac glycoside the k_d value of the type I complex was larger than that of type II, the binding between the sugar moiety and the enzyme in the type I complex appeared less stable than in the type II complex, although an additional functional group, 2′-α-hydroxyl, was able to bind to the enzyme. This might be explained by a difference in the distance between the sugar moiety and the sugar-specific site; i.e., functional groups of the sugar moiety in the type I complex may not be as close to the sugar-specific site on the enzyme as they are in the type II complex.

The activation energy of dissociation in type I complexes did not vary significantly from 30 kcal/mole in the series of cardiac monoglycosides; the value for type II complexes similarly was approximately 20 kcal/mole. As was the case for type II complexes, these results suggest that a conformational change of the enzyme may be involved in the dissociation of type I complexes.

In conclusion, the salient factor in the difference in k_d between type I and type II complexes may be a difference in mode of binding between the sugar moiety of the glycoside and a sugar-specific site on the enzyme, and thus difference may be due to a difference in the conformation of the sugar-specific site.

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