Biochemical Pharmacology, Vol. 33, No. 13, pp. 2089–2099, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

INTERACTION OF CARDIAC GLYCOSIDES AND Na,K-ATPase IS REGULATED BY EFFECTOR-CONTROLLED EQUILIBRIUM BETWEEN TWO LIMIT ENZYME CONFORMERS

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(*Received* 18 *October* 1983; *accepted* 26 *January* 1984)

Abstract—The paper describes the dissociation parameters of the complexes between [^{3}H]-digitoxin and Na,K-ATPase ($Na^+ + K^+$-activated, Mg^{2+}-dependent ATP phosphohydrolase, E.C. 3.6.1.3) from pig cardiac muscle and brain cortex formed and dissociated in the presence of different combinations and concentrations of the enzyme effectors ATP, Mg^{2+}, Na^+ and K^+. Systematic variation of effector-ligation of Na,K-ATPase allowed production of glycoside complexes with two enzyme conformers only, which showed either rapid or slow dissociation kinetics. Appropriate changes of enzyme ligation allowed the interconversion of the two conformer types. Biphasic, rapid and slow glycoside release was not bound with the presence of two Na,K-ATPase isozymes, but caused by the enzyme ligation-determined coexistence of the two conformers of Na,K-ATPase. The rate constants for the rapid and slow glycoside release were within the complexes of each dissociation type much alike indicating uniform isomerization kinetics of the two conformers even when differently liganded. Taken together, the observations indicated the effector-controlled isomerizations of two conformers of Na,K-ATPase possessing different geometries of the glycoside binding domain. Present findings and relevant literature data were integrated in a circular, consecutive and simultaneous model for induced conformation changes that accounted for the regulation of the interaction of cardiac glycosides and Na,K-ATPase through an effector-controlled equilibrium between two limit enzyme conformers.

A great number of variables is known to influence the kinetics of interaction between digitalis compounds and Na,K-ATPase [$Na^+ + K^+$-activated, Mg^{2+}-dependent ATP phosphohydrolase E.C. 3.6.1.3.] as reviewed in references [1–3]. The variables include the source of the enzyme used, the nature, combination and concentration ratio of enzyme effectors applied (particularly ATP, Mg^{2+}, Na^+, K^+), the structure of the digitalis compound tested, and the time of digitalis–enzyme interaction chosen. The diverse findings, which seemed partly to be blurred by instability [4, 5] or vesicular nature [6] of the enzyme preparation and impurity of the labeled digitalis compounds [7], resisted as yet a unifying mechanistic explanation.

Allen *et al.* [8] proposed a two-conformer model of Na,K-ATPase which accounts for the time-dependence of ouabain-inhibition of, and of ouabain-binding to Na,K-ATPase as follows. K^+ reduces the steady-state concentration of an ouabain-receptive conformer. The relatively unstable ouabain–enzyme complex, primarily formed with the receptive conformer, converts slowly to a more stable conformer to which the drug is more tightly bound. Schönfeld *et al.* [9] and Lindenmayer *et al.* [10] proposed the occurrence of an equilibrium between two interconvertible enzyme conformers which explains the influence of effector-ligation of Na,K-ATPase on the kinetics and steady state of ouabain–enzyme interaction. The poise of the equilibrium was assumed to be shifted through ligation with ATP and Na^+, or with ATP, Mg^{2+}, and Na^+ to the side of the conformer showing high ouabain affinity, and through ligation with K^+, with Mg^{2+}, and with Mg^{2+} and Na^+ or K^+ to the side of the conformer showing low or no ouabain affinity. The underlying findings and the derived interpretations were confirmed, accepted and extended in studies by Erdmann and Schoner [11], Bodemann and Hoffman [12], and Wellsmith and Lindenmayer [6].

The outlined model of two interconvertible enzyme conformers, only one of which interacts easily with ouabain, does not seem to explain the observation of curvilinear Scatchard-type plots [4, 7, 11, 13] in which the concentration ratio of bound ouabain: free ouabain is plotted versus the concentration of enzyme-bound ouabain. If namely Na,K-ATPase existed under all conditions of ligand-exposure in a rapidly poising equilibrium between a "high-affinity" and a "low-affinity conformer", ouabain binding would finally take place to the higher affinity conformer only. In the steady state, this conformer should be the only one present so that a straight line would be obtained in the Scatchard plot [7]. Obviously then, curvilinear Scatchard plots indicate the coexistence of at least two enzyme conformers the gradated glycoside affinity of which is apparently due to differing effector-ligation of the

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Abbreviations: P_i, orthophosphate; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate.

enzyme molecules in total population (cf. refs. [4, 7, 11, 13]).

The effector-dependent formation and interconversion of digitalis-receptive enzyme conformers is the central theme of our present study. In the past, the question has been tackled mostly by following the kinetics of ouabain-inhibition of and ouabain-binding to the enzyme [6, 8–12]. However, the binding reaction may show pseudo-first-order kinetics with respect to ouabain concentration even though the dissociation reaction reveals the coexistence of low- and high-affinity conformers characterized by rapid or slow ouabain dissociation rates [14]. We have chosen the dissociation reaction as an analytical method for studying the question of conformer interconvertibility, because it yields more information such as the presence of two separate components of glycoside–enzyme complexes [14].

We have compared Na,K-ATPase preparations from cardiac muscle containing one enzyme form, and from brain cortex containing two enzyme forms with different affinities to strophanthidin, ouabain and digitoxin [15–17]. The comparison should reveal possible differences in the effector-control of glycoside interaction with the isozymes. In previous studies, the dissociation of ouabain complexes has been described to show either monophasic kinetics with enzyme preparations from cardiac muscle [18] and brain [19], but also multiphasic or biphasic kinetics with enzyme preparations from cardiac muscle [18] and brain [14, 18].

A systematic study of the effector-dependence of dissociation parameters as approached in our present investigation has not yet been carried out (for earlier findings cf. ref. [1–3, 8–14, 18–20]). As analytical tools, we have chosen the highly hydrophilic ouabain and the strongly lipophilic digitoxin, because lipid solubility is proposed to play a decisive role in the dissociation rate, which is thus believed to involve a lipid barrier [20]. Since our study revealed surprisingly small differences between the two glycosides concerning the dissociation of their complexes with the Na,K-ATPases from the two sources, we report here essentially on our findings with digitoxin which was hitherto seldom applied.

On the basis of our present findings and of relevant earlier observations, we have derived in this paper a unifying model of induced conformation changes describing the effector influence on the regulation of the interaction between digitalis compounds and Na,K-ATPase. As preliminaries to this endeavour, we had to exclude various putative causes for biphasic dissociation pattern that would affect or forbid the intended model building. To facilitate the realization of the final outcome of the present paper, our findings have been described in terms of the emerging mechanistic model, presented in the discussion part, that depicts the digitalis interaction with either the relaxed enzyme conformer, or the tense enzyme conformer, allowing rapid or slow digitalis release.

MATERIALS AND METHODS

Materials. ATP from Reanal (Budapest) was used as the imidazole salt. [^{3}H]-Digitoxin (5.88 GBq/mmole) from Isocommerz (Berlin, G.D.R.) was labeled on the butenolide ring by hydrogen–tritium exchange [21]. Thin-layer chromatography showed the glycosides free of labeled and unlabeled contaminants. All agents used were of analytical grade.

Na,K-ATPase preparations and activities. The enzymes were little purified on purpose to get undeteriorated, stable preparations of non-vesicular membrane fragments. The preparations used were obtained from pig heart muscle and pig brain cortex essentially by means of the procedures described by Matsui and Schwartz [22] or Samaha [23], respectively. To afford comparability of the preparations, a treatment with NaI as described in ref. [22] was applied as a common final step in both procedures. The Na,K-ATPase activity was determined by incubating 2–4 mg protein of the preparations in 2.5 ml of a 25 mM imidazole–HCl buffer solution pH 7.4 (37°) containing 130 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 5 mM KCl at 37° in the absence or presence of 0.1 mM ouabain. Ouabain totally suppressed ATP hydrolysis which was thus solely due to Na,K-ATPase. The activity of the fresh cardiac or brain preparation calculated from the initial rate was 20 ± 4 μmoles P_i/mg protein per hr and 100 ± 20 μmoles P_i/mg protein per hr, respectively. One unit of enzyme activity (U) was defined as that enzyme quantity which liberated 1 μmole P_i from ATP within 1 hr under the conditions specified. The membrane suspensions were quickly frozen in small aliquots and kept at −20°. They were normally used within 4 weeks after preparation during which their activity did not decrease. The protein content of the membrane preparations was determined according to Lowry *et al.* [24] using bovine serum albumin as reference protein. The P_i was quantified as described by Lohmann and Langen [25].

Standard conditions for formation of the glycoside-complexes with Na,K-*ATPase* (specifications in the legends to figures and Table 1). The glycoside was applied in concentrations below saturation of Na,K-ATPase molecules present in total population. This should ensure full reflection of changes in the interaction parameters due to differing effector-ligations of enzyme sites. The glycoside interaction with the enzyme was promoted by its exposure to ATP, Mg^{2+}, Na^+, and K^+. These enzyme effectors, contrary to the often preferred ligands P_i and Mg^{2+}, induce states of the enzyme which presumably resemble some of those occurring in the *in situ* setting of Na,K-ATPase [18]. The complexes were formed by incubating at 37° for variable time periods the tritiated glycoside with the enzyme preparation in 40 ml of a 25 mM imidazole–HCl buffer solution pH 7.4 (37°) containing in addition to ATP the various enzyme effectors in different combinations and concentrations. The reaction was started by adding the enzyme preparation to the mixture. The value of non-specific binding was assessed in parallel by omitting ATP and $MgCl_2$ from the binding medium. It was equivalent to the value determined in the presence of excess unlabeled ouabain and the effectors applied in the various experiments. The levels of non-specific [^{3}H]-digitoxin binding in relation to saturation binding amounted to 10%. In case of large changes in the concentration of NaCl, choline chlor-

Table 1. Effector-control of the dissociation parameters of [^{3}H]-digitoxin-complexes with Na,K-ATPase from brain cortex and cardiac muscle

Set No.	Effectors (mM)	Cerebral enzyme pmole bound/U	Slow complex (%)	$k_{-1} \cdot 10^2$ (min^{-1}) Rapid	$k_{-2} \cdot 10^2$ (min^{-1}) Slow	Cardiac enzyme pmole bound/U	Slow complex (%)	$k_{-1} \cdot 10^2$ (min^{-1}) Rapid	$k_{-2} \cdot 10^2$ (min^{-1}) Slow
I	10 Na^+, 10 CDTA 0.003 ATP, O K^+	0.9 ± 0.1 (4)	0	9.7 ± 1.6	—	n.i.	n.i.	n.i.	n.i.
II	10 Na^+, 0.2 CDTA 0.003 ATP, O K^+	0.9 ± 0.1 (4)	0	8.5 ± 2.0	—	n.i.	n.i.	n.i.	n.i.
III	10 Na^+, 0.2 CDTA 2 ATP, O K^+	1.5 ± 0.1 (9)	0	11.9 ± 2.5	—	0.3 ± 0.1 (4)	0	8.3 ± 3.6	—
IV	130 Na^+, 0.2 CDTA 2 ATP, O K^+	0.8 ± 0.1 (6)	0	7.3 ± 0.6	—	0.7 ± 0.1 (4)	0	6.5 ± 0.5	—
V	10 Na^+, 4 Mg^{2+} 2 ATP, O K^+	1.7 ± 0.2 (3)	25 ± 5*	8.2 ± 1.0*	1.5 ± 0.3*	1.2 ± 0.1 (4)	54 ± 7*	11.1 ± 1.0*	1.7 ± 0.3*
VI	130 Na^+, 4 Mg^{2+} 2 ATP, O K^+	2.6 ± 0.1 (4)	50 ± 8	7.7 ± 1.0	1.2 ± 0.2	1.9 ± 0.1 (5)	94 ± 2	—	1.1 ± 0.1
VII	130 Na^+, 4 Mg^{2+} 0.003 ATP, O K^+	1.4 ± 0.1 (4)	69 ± 6	10.3 ± 2.0	1.6 ± 0.3	n.i.	n.i.	n.i.	n.i.
VIII	130 Na^+, 4 Mg^{2+} 2 ATP, 5 K^+	3.5 ± 0.7 (4)	75 ± 6*	7.3 ± 2.7*	0.9 ± 0.1	2.7 ± 0.6 (7)	97 ± 4	—	0.7 ± 0.1
IX	130 Na^+, 4 Mg^{2+} 2 ATP, 10 K^+	2.6 ± 0.5 (4)	98 ± 3	—	0.8 ± 0.1	3.2 ± 0.1 (3)	98 ± 4	—	0.4 ± 0.1

The complexes were formed under standard conditions by incubating 2–3 mg protein of the enzyme preparations with 0.1 μM [^{3}H]-digitoxin in presence of the effector sets No. I–VII or with 0.3 μM [^{3}H]-digitoxin in presence of effector sets No. VIII and IX for 20–60 min so that the indicated pmole were bound. At zero time, the [^{3}H]-digitoxin dissociation from the complexes was revealed under standard conditions by adding unlabeled digitoxin to give a final concentration of 50 μM. The percentages of the slowly dissociating [^{3}H]-digitoxin-complexes (Slow), and the dissociation rate constants for the rapidly and slowly dissociating complexes (k_{-1} Rapid and k_{-2} Slow, respectively) are shown. The values represent the arithmetic mean with standard error for the number of assays indicated in parentheses. n.i.: not investigated. *Graphically estimated.

ide was used to maintain osmolarity in both complex formation and dissociation.

Standard conditions for determination of complex dissociation (specifications in the legends to figures and Table 1). The dissociation kinetics were traced with an isotopic dilution method to avoid changes to the basic incubation conditions, since the often preferred alternative procedure (including the cooling of the sample to 0°, the repeated washings of the complex in the centrifuge, the resuspending of the complex in different media, and the rewarming of the complex suspension) can alter the properties of the original complex and make undetectable any easily dissociable complex [26]. Thus, without interruption of pre-incubation at 37°, the kinetics of dissociation of the tritiated glycosides from the complexes with the enzyme were revealed by adding at the chosen time (arbitrarily denoted zero time in the following) excess unlabeled glycoside sufficient to prevent further binding of tritiated glycoside. At various time intervals, 4 ml aliquots were removed from the incubation mixture to separate bound and unbound tritiated glycoside by filtering the membrane suspension through glass fibre filters (Whatman GF/C, 2.3 cm in dia). Accelerated by suction, the filtration of the aliquot and the subsequent washing of the membrane film on the filter with twice 2 ml ice-cold 25 mM imidazole–HCl buffer solution took less than 15 sec. As the amount of [^{3}H]-digitoxin remaining absorbed to the glass fibres was relatively high, the membrane film, removed after drying, was transferred to glass vials containing 10 ml scintillation fluid [27] and 1 ml water. The counting efficiency monitored by the external channel ratio method was 50%. To determine the amount of tritiated glycoside bound at zero time, aliquots were removed from the incubation medium either immediately before or 15 sec after adding excess unlabeled glycoside. The results were indistinguishable. All experiments presented in Fig. 1–7 and Table 1 were repeated at least twice, mostly three or more times showing similar results. The data points taken in duplicate were corrected for the protein loss from filtration and for the non-specific binding of the tritiated glycoside. The results of a number of assays (cf. Table 1) are given as the arithmetic means with standard deviations.

Presentation and mathematical treatment of experimental data. To facilitate comparison of the influence of different effector combinations and concentration on the dissociation kinetics, the amounts of tritiated glycoside remaining bound after various time intervals were normalized by expressing them as percentages of the glycoside amount bound at zero time and represented in the form of semi-logarithmic dissociation plots. As depicted in Figs. 1 to 7, the glycoside–enzyme complexes formed under various conditions showed monophasic-rapid or monophasic-slow dissociation kinetics, and biphasic, rapid and slow dissociation kinetics, respectively. Initial estimates for the parameters to be optimized were obtained by graphical techniques. As ordinarily accomplished [28, 29], a regression line, fitted by least-squares methods to the slow exponential, was extrapolated to zero time. The difference between observed values and those on the extrapolated regression line was replotted yielding a straight line. This finding indicated that the observed dissociation curve of the glycoside–enzyme complexes could be accounted for by two independent exponential components, each having different rate constants for dissociation. The dissociation rate constant and the relative proportion of each component were calculated from slopes and ordinate intercepts of regression lines. The data points properly defining a straight line with steep or flat slope were subjected to a computerized linear regression analysis working with an error probability of $\alpha = 0.05$. The routine, stored and executed on a Hewlett Packard 9815, provided the percentages of the rapidly and slowly dissociating complex and the corresponding dissociation rate constants. The results are given in Table 1.

RESULTS

1. Preliminaries

The biphasic dissociation pattern observed ([14, 18, 19] and present paper) could have been caused by several circumstances, extraneous to the coexistence of two enzyme conformers, that were therefore initially checked.

Anticooperative interaction between two digitalis binding domains in the oligomeric enzyme molecule was not involved because its three characteristic kinetic consequences [30] could not be traced. First, the digitoxin-complexes of Na,K-ATPase from brain cortex dissociating in the presence of Na^+, ATP and K^+ but absence of free Mg^{2+} (Fig. 1) or in the presence of Na^+, Mg^{2+} and ATP, and of Na^+, Mg^{2+}, ATP and K^+ (Fig. 2) exhibited, both at low and high degrees of digitalis occupation of enzyme molecules present, analogous biphasic dissociation patterns. Second, higher degrees of digitalis-saturation of enzyme molecules did not result in an increase of the percentage of the rapidly dissociating digitalis-complex. Due to conformer transition (see below), just the reverse was experimentally found when the higher occupancy of enzyme molecules was produced, on the one hand, by prolongation of the time period for complex formation in the presence of Na^+, Mg^{2+}, ATP and K^+ (Fig. 1) or, on the other hand, by raising the glycoside concentration for complex formation in the presence of Na^+, Mg^{2+} and ATP (Fig. 2). Third, at low occupancies large increases of medium glycoside concentration, produced by isotopic dilution, did not markedly accelerate the dissociation of bound glycoside molecules from the complex to be expected in case of an anticooperative destabilization. Up to 50,000-fold increases of medium glycoside concentration did likewise not change the biphasic dissociation pattern of the digitoxin-enzyme complexes (Figs. 1 and 2). Taken together, the results of the three approaches let conclude that anticooperative interaction between two digitalis binding domains on the same enzyme molecule did not underly the observed biphasic dissociation kinetics. Of course, absolute half-of-the-sites or general all-of-the-sites occupation of digitalis binding domains (cf. refs. [31, 32]), that both would show monophasic dissociation, were not

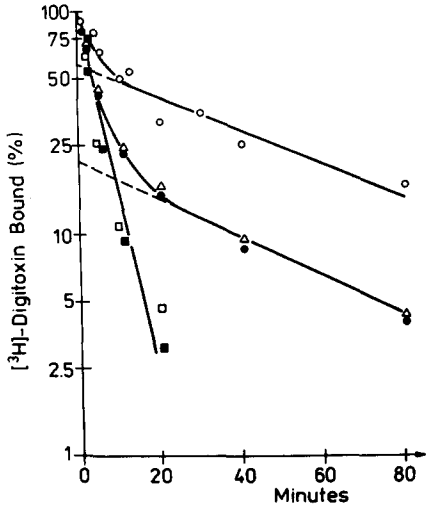

Fig. 1. Monophasic-rapid and biphasic dissociation of [^{3}H]-digitoxin-complexes of Na,K-ATPase from brain cortex when pre-incubation time, [^{3}H]-digitoxin-occupancy and K^+-exposure of enzyme molecules, and procedure for revealing the [^{3}H]-digitoxin release were varied. Certain complexes were formed by pre-incubating in 5 mM imidazole–HCl buffer solution (pH 7.4) 5 mg protein with 0.1 μM [^{3}H]-digitoxin in the presence of 130 mM NaCl, 4 mM $MgCl_2$ and 2 mM ATP for 0.5 min resulting in lower occupancy (0.9 pmole [^{3}H]-digitoxin/U) or for 40 min resulting in higher occupancy (1.9 pmole [^{3}H]-digitoxin/U). The release of [^{3}H]-digitoxin to the medium was revealed in the enzyme populations with both lower occupancy (■) and with higher occupancy (□) during continued incubation through tenfold dilution with the buffer solution containing 4 mM CDTA and 100 μM unlabeled digitoxin. The other complexes were formed by incubating under otherwise similar conditions 5 mg protein with 0.1 μM [^{3}H]-digitoxin in the presence of 130 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP and 10 mM KCl for 1 min resulting in lower occupancy (0.6 pmole [^{3}H]-digitoxin/U) or for 40 min resulting in higher occupancy (1.0 pmole [^{3}H]-digitoxin/U). The release of [^{3}H]-digitoxin to the medium in the enzyme populations with lower occupancy (●,△) or with higher occupancy (○) was revealed during continued incubation through tenfold dilution with the buffer solution containing 4 mM CDTA (△) or additionally 100 μM unlabeled digitoxin (●,○).

excluded, but these enzyme reactivities, if present, were irrelevant to the theme of the present paper.

Rebinding of labeled glycoside molecules, released from the complex with Na,K-ATPase to the bulk medium, was not involved in non-linear dissociation pattern, since the observed biphasic dissociation kinetics remained unaltered when rebinding was suppressed by Mg^{2+} chelation with CDTA and chemical dilution, or by Mg^{2+} chelation with CDTA and chemical plus isotopic dilution (Fig. 1) or by isotopic dilution alone (Fig. 2). The finding that the additional isotopic dilution did not affect the dissociation pattern and rate showed that the addition of excess unlabeled glycoside, used as standard procedure in the present paper, is an appropriate method for disclosing the dissociation of tritiated glycoside from the various complexes with the enzyme.

Relevant changes of the concentration or nature of enzyme effectors during the formation and dissociation of the enzyme–glycoside complexes did not appear to be involved in the occurrence of non-linear dissociation kinetics as the following four lines of evidence revealed.

First, the biphasic dissociation kinetics of the complexes formed between digitoxin and cerebral Na,K-ATPase in the presence of Na^+, Mg^{2+}, ATP and $\pm K^+$ existed irrespective of whether the ATP concentration remaining at the start of complex dissociation was not much below 2 mM or was above 10 μM (Figs. 1 and 2). The complex of digitoxin with Na,K-ATPase from brain cortex formed in the presence of Na^+, Mg^{2+} and very low ATP concentration showed similar biphasic dissociation patterns with similar large and small dissociation rate constants irrespective of whether ATP was added at the initiation of complex dissociation or not to yield

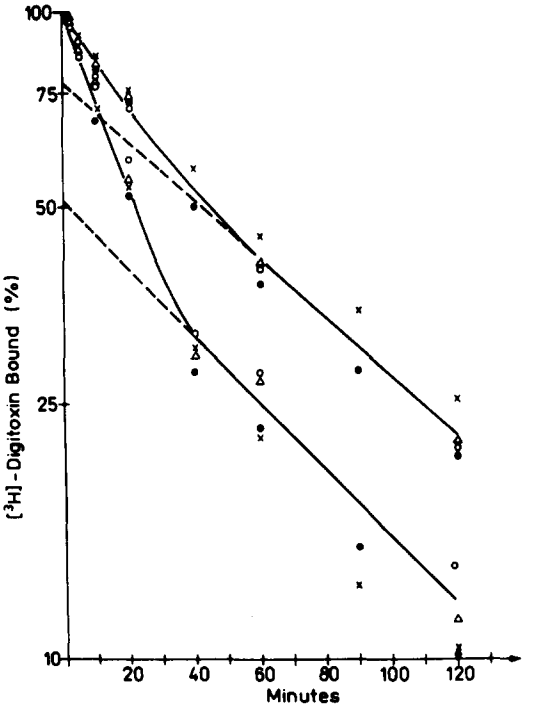

Fig. 2. Biphasic dissociation of [^{3}H]-digitoxin-complexes of Na,K-ATPase from brain cortex when the [^{3}H]-digitoxin concn, the [^{3}H]-digitoxin-occupancy and K^+-exposure of enzyme molecules were varied. Lower data sets: the complexes were formed under standard conditions by pre-incubating 2–3 mg protein in the presence of 130 mM NaCl, 4 mM $MgCl_2$ and 2 mM ATP for 90 min with 0.001 μM (△), 0.005 μM (○) or 0.01 μM (X) [^{3}H]-digitoxin, and for 30 min with 0.3 μM (●) [^{3}H]-digitoxin yielding 0.2 pmole (△), 0.7 pmole (○), 1.6 pmole (X) and 3.6–4.6 pmole (●) [^{3}H]-digitoxin bound/U. Upper data sets: the complexes were formed under standard conditions in the presence of 130 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP and 5 mM KCl, otherwise as indicated above, yielding 0.1 pmole (△), 0.4 pmol (○), 1.0 pmole (X) and 3.6–4.8 pmole (●) [^{3}H]-digitoxin bound/U. Depending on the [^{3}H]-digitoxin concn chosen and on the absence or presence of K^+ during pre-incubation, the ATP concn at zero time were estimated to range not far below 2 mM (K^+ absence) or not much above 10 μM (K^+ presence). In each case, the [^{3}H]-digitoxin release was revealed under standard conditions by adding unlabeled digitoxin to give a final concn of 50 μM.

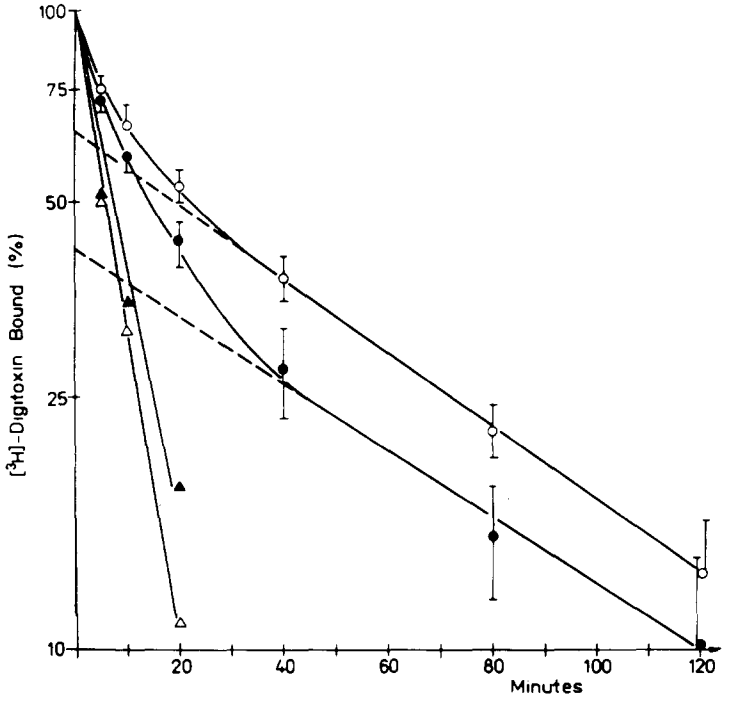

Fig. 3. Biphasic, rapid and slow dissociation of [^{3}H]-digitoxin-complexes of Na,K-ATPase from brain cortex when the ATP concn was varied. The complexes were formed under standard conditions by pre-incubating 0.1 μM [^{3}H]-digitoxin with 2–3 mg protein in the presence of 130 mM NaCl, 4 mM $MgCl_2$ and 0.003 mM ATP for 20 min yielding 1.4 ± 0.1 pmole [^{3}H]-digitoxin bound/U. The [^{3}H]-digitoxin release was revealed under standard conditions by adding unlabeled digitoxin to give a final concn of 50 μM (○;△ rapid component of curve) or adding unlabeled digitoxin and ATP to give final concn of 50 μM and 2 mM, respectively (●;▲ rapid component from it).

a high ATP concentration (Fig. 3). These results are in line with the fact that the rate of glycoside binding is maximized already at ATP concentrations near 10 μM with no further increase up to 800 μM ATP [33].

Second, the biphasic dissociation did not result from the coexistence of enzyme molecules unliganded and liganded with ADP, because biphasicity was invariably observed when ADP was not produced due to the absence of Mg^{2+} (Fig. 6) or when small or great amounts of ADP were released through ATP hydrolysis due to short or long incubation times (Fig. 1) or due to incubation in the absence or the presence of K^+ (Fig. 2).

Third, the biphasic dissociation pattern was presumably not caused by differences in the rates of the release of the glycosides from coexisting complexes with the phosphoenzyme and dephosphoenzyme. These two enzyme states, in part initially present during complex formation, no longer coexisted during complex dissociation as could be inferred from the following information. Ouabain binding to the enzyme is known to result in a decrease of the steady-state level of the phosphoenzyme which is well correlated with an increase of the level of bound ouabain [34]. The rate of the release of P_i from the ouabain–phosphoenzyme complex is much more rapid than the dissociation of ouabain from the ouabain–dephosphoenzyme complex; there is no rephosphorylation from ATP until ouabain has also been released from the dephosphoenzyme [35]. Although the ouabain–phosphoenzyme complexes are insensitive to K^+ with respect to the dephosphorylation rate [36], Na,K-ATPase is sensitive to K^+ with respect to the percentage of the rapidly and slowly dissociating complexes irrespective of whether a phosphorylated intermediate could have been formed due to the presence of Mg^{2+} or could not due to the absence of Mg^{2+} (Figs. 3 and 4 vs Figs. 5 and 6). Hence, the phosphorylation–dephosphorylation cycle of the enzyme and the dissociation pattern of the glycoside–complexes are independent processes. A similar conclusion was drawn earlier from independent observations [9]. The available information suggests that the glycoside dissociated from the complex with the dephosphoenzyme and that the dissociation kinetics were decided by the presence of two conformers of the enzyme determined in part by the absence or presence of K^+ binding to the enzyme.

Fourth, the glycoside–enzyme complex obtained in the presence of Na^+, Mg^{2+}, ATP and K^+ could not have been a mixture of complexes formed through ATP- and P_i-supported binding since P_i fails to support glycoside binding in the presence of the Na^+ concentrations applied [18].

Finally, the biphasic dissociation did not appear to result from a mixture of native enzyme molecules and of enzyme molecules altered as to the interaction with the glycoside during complex formation or enzyme preparation (cf. refs. [4, 5]). Actually, after chemical dilution of the tritiated glycoside, the

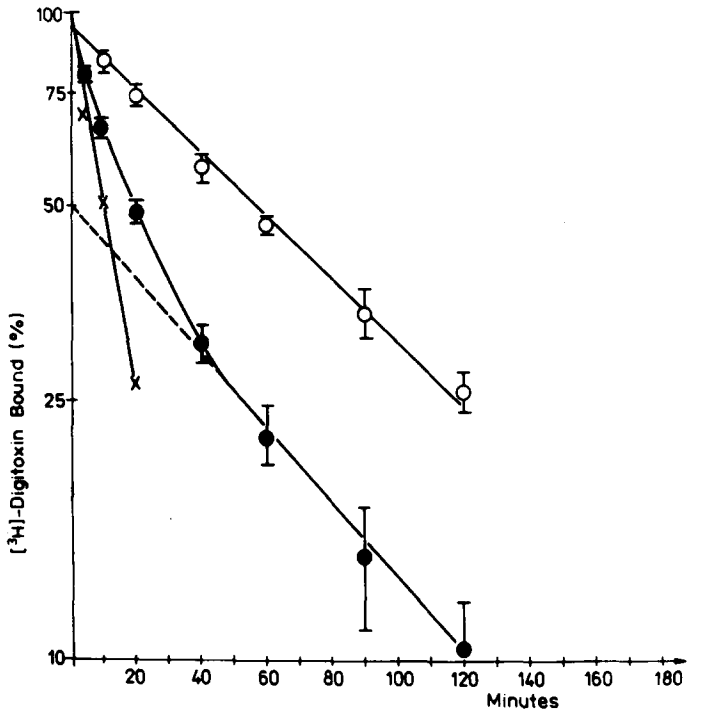

Fig. 4. Monophasic slow and biphasic, rapid and slow release of [^{3}H]-digitoxin from complexes with Na,K-ATPase when cardiac and cerebral enzyme, respectively were studied. The complexes were formed by pre-incubating 0.3 μM [^{3}H]-digitoxin with 7 mg protein of cardiac preparation or 3 mg protein of cerebral preparation in the presence of 130 mM NaCl, 4 mM $MgCl_2$ and 2 mM ATP for 30 min yielding in each case 3.9 ± 0.6 pmole [^{3}H]-digitoxin bound/U. The release of [^{3}H]-digitoxin from the complexes with the cardiac enzyme (○) or the cerebral enzyme (●; X rapid component of curve) was revealed by adding unlabeled digitoxin to give a final concn of 50 μM.

recovered enzyme activity corresponded to the proportion of the uncomplexed enzyme. Moreover, the same enzyme preparations, showing under certain ligand exposures biphasic dissociation of the complexes, yielded in the presence of the appropriate ligands glycoside-complexes exhibiting monophasic-rapid or monophasic-slow dissociation kinetics (see below). The latter results also made it unlikely that the biphasicity resulted from inhomogeneities in the radiochemical composition of the [^{3}H]-digitoxin. Since both the hydrophilic ouabain (not demonstrated) and the lipophilic digitoxin showed biphasic dissociation, preferentially from complexes with cerebral Na,K-ATPase (Fig. 4; Table 1, sets V to VIII), this kinetic pattern could not be a matter of the lipophilicity of the digitalis compounds, although lipid solubility seems to have some influence on the characteristics of their interaction with Na,K-ATPase (cf. ref [20] and below).

2. Monophasic-rapid complex dissociation

The complexes of digitoxin with cerebral and cardiac Na,K-ATPase showed monophasic-rapid dissociation in the absence of enzyme-bound Mg^{2+}. CDTA interrupts, within less than 1 sec, completely phosphorylation of Na,K-ATPase from ATP through reduction of the free Mg^{2+} level by four orders of ten [37]. Hence, we used CDTA to study the influence of a virtual absence of Mg^{2+} on the complex dissociation which was characterized by the following two findings. First, the digitoxin-complexes with both cerebral and cardiac enzyme, formed in the presence of ATP, CDTA and additionally either low [Na^+] (Figs. 5–7; Table 1, sets I to III) or high [Na^+] (Figs. 5–7; Table 1, set IV), showed monophasic-rapid dissociation. The amount and the dissociation pattern of the complexes were not altered when the enzymes were preincubated with 0.2 or 10 mM CDTA up to 30 min at 37° (not demonstrated). Second, the digitoxin-complexes formed with the cerebral enzyme in the presence of ATP, Mg^{2+} and Na^+, otherwise showing biphasic glycoside release (Fig. 2) exhibited monophasic-rapid dissociation after admixture of CDTA together with the termination of labeled glycoside binding (Fig. 1).

3. Monophasic-slow complex dissociation

In the absence of Mg^{2+}, the admixture of 10 mM K^+ at the start of dissociation analysis produced a strong reduction of dissociation rate in the presence of 10 mM, but not of 130 mM Na^+ (Fig. 7). Thus, the stabilizing effect of K^+ appeared to result from the K^+-occupation of the K^+ activation sites, which was competitively prevented by high [Na^+].

The presence of Mg^{2+} during complex formation considerably enhanced the percentage of the slowly dissociating complex formed between either enzyme and digitoxin in the presence of ATP and low [Na^+] (Table 1, set III vs V) as well as high [Na^+] (Table

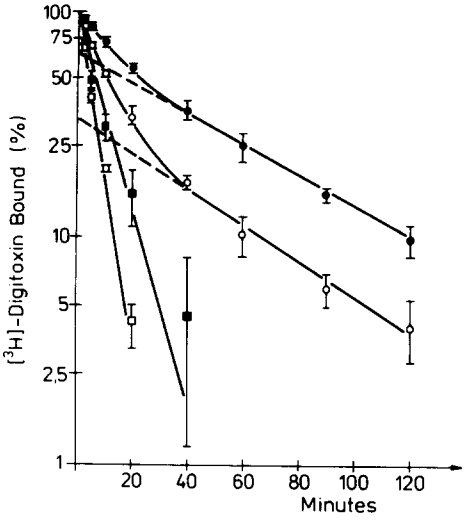

Fig. 5. Monophasic-rapid and biphasic, rapid and slow release of [^{3}H]-digitoxin from complexes with Na,K-ATPase from brain cortex when enzyme exposures to Mg^{2+} and Na^+ were varied. Certain complexes were formed by pre-incubating 2–3 mg protein in the presence of 10 mM NaCl, 0.2 mM CDTA and 2 mM ATP for 20 min yielding 1.5 ± 0.1 pmole [^{3}H]-digitoxin bound/U. The release of [^{3}H]-digitoxin was revealed by adding unlabeled digitoxin to give a final concn of 50 μM (□) or unlabeled digitoxin and NaCl to give final concn of 50 μM and 130 mM, respectively (■). The other complexes were formed by pre-incubating 2–3 mg protein in the presence of 10 mM NaCl, 4 mM $MgCl_2$ and 2 mM ATP for 20 min yielding 1.7 ± 0.2 pmole [^{3}H]-digitoxin bound/U. The release of [^{3}H]-digitoxin was revealed as given above by adding unlabeled digitoxin (○) or unlabeled digitoxin and 130 mM NaCl (●).

1, set IV vs VI), although at high [Na^+] the increment was larger. The admixture of Mg^{2+} at the start of dissociation analysis produced at low or high [Na^+] a small or a large reduction of dissociation rate, respectively (Fig. 6).

The increase of the Na^+ concentration from 10 to 130 mM enlarged the percentage of the slowly dissociating complex between either enzyme and digitoxin formed in the presence of Mg^{2+}, ATP and Na^+ (Fig. 5; Table 1, set V vs set VI). At the high concentration, Na^+ appeared to favor the slowly dissociating complex by occupying besides the Na^+ also the K^+ activation sites.

4. Biphasic glycoside release

The Na,K-ATPase preparations from both brain cortex and cardiac muscle formed glycoside–enzyme complexes in relaxed and tense conformation allowing rapid or slow dissociation, respectively, but the prerequisites of effector-ligation of the isozymes for their occurrence were partially different.

The cardiac enzyme preparation adopted both conformations with digitoxin in the presence of Mg^{2+}, ATP and low [Na^+] (Table 1, set V). However, unlike the cerebral enzyme, the cardiac enzyme adopted only tense conformation in the presence of either Mg^{2+}, ATP and high [Na^+] (Fig. 4; Table 1, set VI) or Mg^{2+}, ATP, K^+ and high [Na^+ (Table 1, sets VIII and IX).

The cerebral enzyme preparation yielded rapidly and slowly dissociating complexes with digitoxin under most conditions studied, such as in the presence of Mg^{2+}, ATP and additionally low [Na^+] (Fig. 4; Table 1, set V), high [Na^+] (Figs. 2 and 4; Table 1, sets VI and VII) or high [Na^+] and K^+ (Fig. 2; Table 1, set VIII). The coexistence of the two glycoside-complexes required during complex dissociation besides ATP and Na^+ either the presence of Mg^{2+} (Figs. 2 and 6) or, if Mg^{2+} was absent, the presence of K^+ (Figs. 1 and 7).

In conclusion, the two cerebral enzyme forms adopted as the cardiac enzyme form with certain effector-ligations either only relaxed conformers allowing rapid glycoside release or only tense conformers allowing but slow glycoside release. As further shown, the occurrence of biphasic dissociation of digitoxin-complexes with the cardiac and cerebral enzyme preparation was not bound with the presence of two enzyme forms having different, structurally fixed glycoside binding domains, but indicated the effector-determined coexistence of relaxed and tense conformers of Na,K-ATPase that allowed rapid or slow glycoside release, respectively. The major difference between the two isozymes seemed to be the differential affinities of the Na^+ activation sites and the K^+ activation sites to Na^+ and K^+ (cf. Table 1, set V vs VI, and Fig. 7A vs B).

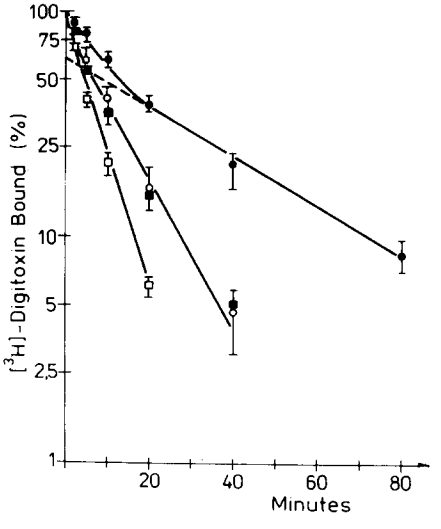

Fig. 6. Monophasic-rapid or biphasic, rapid and slow release of [^{3}H]-digitoxin from complexes with Na,K-ATPase from brain cortex when enzyme exposures to Na^+ and Mg^{2+} were varied. Certain complexes were formed by pre-incubation of 0.1 μM [^{3}H]-digitoxin with 2–3 mg protein in the presence of 10 mM NaCl, 0.2 mM CDTA and 2 mM ATP for 20 min yielding 1.5 ± 0.1 pmole [^{3}H]-digitoxin bound/U. The release of [^{3}H]-digitoxin was revealed by adding unlabeled digitoxin (□) or unlabeled digitoxin and $MgCl_2$ (■) to give final concn of 50 μM and 4 mM, respectively. The other complexes were formed under otherwise similar conditions in the presence of 130 mM NaCl, 0.2 mM CDTA and 2 mM ATP yielding 0.8 ± 0.1 pmole [^{3}H]-digitoxin bound/U. The release of [^{3}H]-digitoxin was again revealed by adding unlabeled digitoxin (○) or unlabeled digitoxin and $MgCl_2$ (●).

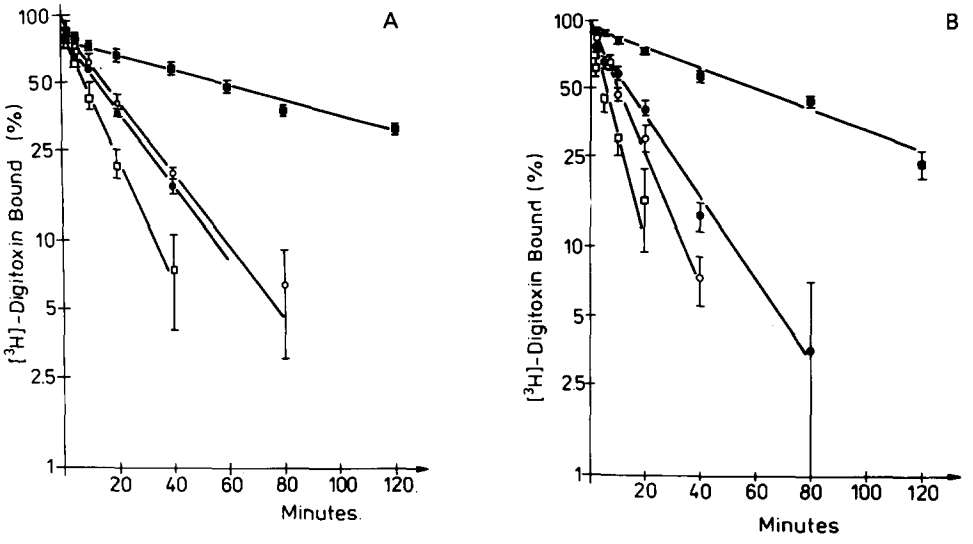

Fig. 7. Monophasic-rapid and monophasic-slow dissociation of [^{3}H]-digitoxin-complexes of Na,K-ATPase from brain cortex (A) or cardiac muscle (B) when enzyme exposures to Na^+ and K^+ were varied. (A) The complexes were formed by pre-incubation of 0.1 μM [^{3}H]-digitoxin with 2–3 mg protein for 20 min in the presence of either 10 mM NaCl, 0.2 mM CDTA and 2 mM ATP yielding 1.5 ± 0.1 pmole [^{3}H]-digitoxin bound/U (□,■), or 130 mM NaCl, 0.2 mM CDTA and 2 mM ATP yielding 0.8 pmole [^{3}H]-digitoxin bound/U (○,●). The release of [^{3}H]-digitoxin was revealed by adding unlabeled digitoxin (□,○) or unlabeled digitoxin and KCl (●,■) to give final concn of 50 μM and 10 mM, respectively. (B) The complexes were formed by pre-incubation of 0.1 μM [^{3}H]-digitoxin with 7–10 mg protein for 20 min in the presence of either 10 mM NaCl, 0.2 mM CDTA and 2 mM ATP yielding 0.3 ± 0.1 pmole [^{3}H]-digitoxin bound/U (□,■), or 130 mM NaCl, 0.2 mM CDTA and 2 mM ATP yielding 0.7 ± 0.1 pmole [^{3}H]-digitoxin bound/U (○,●). The release of [^{3}H]-digitoxin was again revealed by adding unlabeled digitoxin (□,○) or unlabeled digitoxin and KCl (●,■).

5. Uniformity of the various enzyme–glycoside complexes exhibiting rapid or slow dissociation kinetics

As shown in Table 1 for digitoxin (for ouabain not demonstrated), the k_{-1} values of the rapidly dissociating complexes between cerebral enzyme and digitoxin (on an average 0.089 min^{-1}) or ouabain (0.085 min^{-1}) as well as between cardiac enzyme and digitoxin (0.086 min^{-1}) or ouabain (0.041 min^{-1}) did not differ within each series, although the underlying complexes were formed and dissociated in the presence of different combinations and concentrations of the effectors of Na,K-ATPase. As also shown in Table 1 for digitoxin (for ouabain not demonstrated), the k_{-1} values of the slowly dissociating complexes between cerebral enzyme and digitoxin (on an average 0.012 min^{-1}) or ouabain (0.009 min^{-1}) as well as between cardiac enzyme and digitoxin (0.01 min^{-1}) or ouabain (0.008 min^{-1}) did not differ significantly within each series although the underlying complexes were formed and dissociated again in the presence of different effector combinations and concentrations. Hence, the response of a given population of Na,K-ATPase molecules to variations of effector-ligation could be accounted for in terms of the occupancy of only two conformation states. In these states, the distribution of peptide chains was apparently restricted by cooperative transitions such that the glycoside–enzyme complexes allowing rapid or slow glycoside release represented limiting cases of two highly cooperative protein transitions (cf. ref. [39]). Needless to say, the dissociation rate constants of the complexes were determined by both enzyme and glycoside. So, the k_{-1} and k_{-2} values were with digitoxin always somewhat greater than with ouabain. Structurally more divergent digitalis derivatives may be expected to show much greater differences in the dissociation rate constants of their complexes with Na,K-ATPase.

6. Interconversion of the two conformation states

The proportion of enzyme–glycoside complexes in the tense conformation, formed between digitoxin and cerebral enzyme in the presence of Na^+, ATP and Mg^{2+}, became almost "immediately" and completely converted to enzyme–glycoside complexes in the relaxed conformation (allowing rapid dissociation) when at the start of the dissociation analysis Mg^{2+} was chelated with CDTA (cf. Figs. 1 and 2). Even in the presence of Mg^{2+}, a certain percentage of tense glycoside–enzyme conformers was rapidly converted to relaxed conformers by the exposure to high [ATP] (Fig. 3; Table 1, set VII vs VI) or by exposure to low [Na^+] (Fig. 5; Table 1, set V vs VI).

A proportion of enzyme–glycoside complexes in the relaxed conformation, formed between either enzyme and digitoxin in the presence of low [Na^+] and ATP, but absence of Mg^{2+} and K^+, became without significant delay converted to enzyme–glycoside complexes in the tense conformation (allowing slow dissociation) when K^+ was added at the start of the dissociation analysis (Fig. 7). Likewise, a certain percentage of relaxed glycoside–enzyme conformers, formed between digitoxin and cerebral

enzyme in the presence of high [Na^+] and ATP or in the presence of low [Na^+], ATP and Mg^{2+}, was rapidly converted to tense glycoside-enzyme conformers when at the start of the dissociation analysis Mg^{2+} was added (Fig. 6) or [Na^+] was raised (Fig. 5).

DISCUSSION

In the present study, various glycoside–enzyme complexes were formed and dissociated in the presence of different combinations and concentrations of four effectors of Na,K-ATPase. The enzyme preparation of both cerebral cortex and cardiac muscle yielded two types of complexes characterized by rapid or slow glycoside release. The dissociation rate constants differed by one order of ten. Thus, the two types of complexes could easily be differentiated even when instantaneously formed. The findings can fully be described in terms of the circular, consecutive and simultaneous model for effector-induced conformation changes (cf. refs. [6, 39, 40]):

$$\begin{array}{ccc} D + E_R & \rightleftharpoons & E_T + D \\ \downdownarrows\uparrow & & \uparrow\downarrow \\ E_RD & \rightleftharpoons & E_TD \end{array}$$

By definition, E_RD symbolizes the complex of a digitalis compound (D) with the relaxed enzyme conformer (E_R) allowing rapid digitalis release, and E_TD symbolizes the digitalis-complex with the tense enzyme conformer (E_T) allowing slow digitalis release. The equilibria $E_R \rightleftharpoons E_T$ and $E_RD \rightleftharpoons E_TD$ could both be poised by variation of the combination and concentration of the effectors. Complex formation in the presence of ATP and Na^+ and dissociation under the same conditions revealed the formation of E_RD only, whereas dissociation in the additional presence of K^+ or Mg^{2+} disclosed extensive conversion of E_RD to E_TD. Complex formation in the presence of ATP, Mg^{2+} and Na^+ and dissociation under same condition revealed the coexistence of E_RD and E_TD, whereas dissociation in the presence of enhanced [Na^+] or increased [ATP] disclosed partial conversion of E_RD to E_TD or E_TD to E_RD, respectively; dissociation after removal of Mg^{2+} showed complete conversion of E_TD to E_RD. Thus, the type of the primarily formed complex was regulated by the effector-controlled equilibrium $E_R \rightleftharpoons E_T$, and the type of the finally dissociating complex was eventually regulated by the effector-controlled equilibrium between $E_RD \rightleftharpoons E_TD$.

The relevant literature data can be integrated in the framework of the model. As reviewed elsewhere [41], a given population of Na,K-ATPase molecules may consist of a mixture of relaxed and tense enzyme conformers. The [E_R]:[E_T] ratio is increased by exposure to ATP and Na^+, and decreased by exposure to Mg^{2+} and K^+ [41]. Under physiological conditions, i.e. in the presence of ATP, Mg^{2+}, Na^+ and K^+, E_R and E_T symbolize enzyme states in which Na^+ or K^+, respectively, occupy the Na^+ activation sites at the intracellular enzyme surface. Thus, the poise of the equilibrium $E_R \rightleftharpoons E_T$ is a function of the $[Na^+]_i$:$[K^+]_i$ ratio. The influence of Na^+- or K^+-ligation of Na,K-ATPase is manifest beyond the cation-protein interface, and extends probably through the entire catalytic protein. The opposite effect of Na^+ and K^+ on digitalis interaction with Na,K-ATPase reflects cation-induced alterations in the geometry of the digitalis binding domain [1]. Thus, the thermodynamic parameters for the digitalis binding to Na,K-ATPase are widely determined by [Na^+] and [K^+].

The thermodynamic quantities calculated for the dissociation constant of the Na^+ complex with Na,K-ATPase are: $\Delta H^{O'} = 4.1$ kcal/mole and $\Delta S^{O'} = 21.7$ cal/mole-degree [42]. The Na^+ binding to the enzyme is thus characterized by an increase of enthalpy and is driven by a large increase of entropy, i.e. it involves an increase of peptide-chain mobility and decrease of protein "tensity" (cf. ref [39]). The corollary is a low Gibbs activation energy for digitalis binding to E_R and digitalis release from E_RD. Hence, the thermodynamic parameters appear to give an explanation for the rapid formation (see below) and rapid dissociation (this paper) of E_RD.

The thermodynamic quantities calculated for the dissociation constant of the K^+ complex with the enzyme are: $\Delta H^{O'} = -13.6$ kcal/mole and $\Delta S^{O'} = -27.1$ cal/mole-degree [42]. K^+ binding to the enzyme is thus an enthalpy-driven process, and associated with a large decrease of entropy, i.e. it involves an increase of "rigidity" or "tensity" of enzyme protein (cf. ref. [39]). The corollary is a high Gibbs activation energy for digitalis binding to E_T and for digitalis release from E_TD. Hence, the thermodynamic parameters appear to give an explanation for the difficult binding of digitalis to E_T (see below) and for the slow decomposition of E_TD (this paper).

In the presence of ATP and Mg^{2+}, raising [Na^+] increases and raising [K^+] decreases the rate of ouabain interaction with Na,K-ATPase [9, 43]. Kinetic analyses of the ouabain binding rate suggest that Na^+ and K^+ compete for a common site [43]. Na^+ and K^+ modulate that fraction of Na,K-ATPase molecules which manifests the primarily receptive conformation for interaction with the glycoside [43], i.e. E_R in present terminology. Addition of high [Na^+] in the presence of ATP, Mg^{2+} and K^+ promotes ouabain binding to and inhibition of the enzyme [8]. The underlying mechanism appears to be an increase of the [E_R] : [E_T] ratio through inductive conformation change.

The apparent association rate constant for binding of low [ouabain] to Na,K-ATPase, observed in the presence of ATP, Mg^{2+} and Na^+, becomes decreased in the additional presence of K^+ by up to an order of ten [9–14]. The K^+ effect is caused by a reduction of concentration of the primarily ouabain-binding conformation [9], i.e [E_R] in the present terminology. At low [D], E_R appears to be the principal binding conformation. At medium [D], the binding reaction may follow pseudo-first-order kinetics even though the dissociation analysis reveals, instead of the expected monomolecular course, biphasic kinetics [14]. This indicates, besides the presence of E_RD, the gradual formation of E_TD as shown by Allen *et al.* [8] and confirmed in present study. The route taken for the formation E_TD is likely be $D + E_R \rightleftharpoons E_RD \rightleftharpoons E_TD$. At high [D], the K^+ effected decrease of the binding rate and the equilibrium value of digitalis–enzyme complex may be overcome

[9, 14]. Obviously, digitalis binds then also directly to E_T.

Non-linear Scatchard plots referred to in the introduction part [4, 7, 11, 13] cannot be accounted for in the framework of the circular model. They appear to be caused by the coexistence of at least two isozymes of Na,K-ATPase or two conformation states of a single enzyme form which do not equilibrate.

Our study confirms the more general understanding that the biological machinery for regulation is predominantly through conformation control [40]. The rather selective binding of digitoxin to one of the two conformers of Na,K-ATPase carries the necessary implication that the stereoelectronic parameters of the digitalis binding domain are different in the two conformations. This may constitute one of the major promises for successful synthetic further development of cardiotonic steroid glycosides.

Acknowledgements—We wish to thank Mrs. Heidemarie Kistel for technical assistance, Mrs. Ines Schulze for bibliographical and secretarial work in the preparation of the paper, Dr. Werner Schönfeld for fruitful discussions, and Dr. Karla Köpke for help in the computerized evaluation of the experimental data.

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