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Membrane receptor calorimetry: cardiac glycoside interaction with Na,K-ATPase

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Abstract

The receptor–ligand interaction between the cardiac glycoside Ouabain and purified, membrane-bound as well as micellar Na,K-ATPase is investigated. Calorimetric titrations are carried out with micromolar concentrations of the phosphorylated protein in the presence of Mg^{2+}. The measured heat changes provide evidence for an exothermic, high affinity and specific receptor binding process as well as for a low affinity, nonspecific binding to the lipid part of the nanoparticulate membrane fragments. The degree of lipid binding markedly depends on the lipid composition of the tissue. The measured time course of the heat change resulting from specific binding to the receptor site is unusually slow and is limited by the binding kinetics of the ligand. A course estimation of the Ouabain binding kinetics leads to a rate constant around 10^4 mol^{-1} l s^{-1}. Receptor binding is characterized by affinities ranging between 10^7 and 10^8 mol^{-1} l, ΔH values around -95 kJ mol^{-1} and ΔS values of about -130 J K^{-1} mol^{-1} at 25°C. The enthalpic contribution is assumed to be mainly due to hydrogen bond formations between the ligand and the receptor site whereas the large, negative entropy change may be attributed to an increased interaction between water and the protein as a consequence of a conformational transition. The evaluation of the titrations provides stoichiometric coefficients around 0.55, which implies that only about 50–60% of the Na,K-ATPase protomers are capable to bind the cardiotonic steroid. This result is consistent with radioactive phosphorylation studies and appears to be a typical feature of kidney-type Na,K-ATPase preparations. Possible implications of this finding are discussed. As a general result, this study demonstrates how simple and suitable calorimetric titrations with micromolar protein concentrations can be for the purpose of a quantitative characterization of a receptor in nanoparticulate membrane systems.

Keywords: Cardiac glycosides; Cardiotonic steroids; Ouabain; Na,K-ATPase; Membrane receptor; Lipid binding; Calorimetry

1. Introduction

Pharmacological receptors are intracellular or membrane-bound proteins which induce a particular response upon the reversible and specific binding of a ligand. Such receptor molecules have two main functions, namely the recognition of a signal leading to the formation of the receptor–ligand complex, and subsequently the transduction and processing of the signal, which will then provide the corresponding cellular response. Agonist type of ligands can induce a stimulation of the receptor, non-stimulating ones act as antagonists. Receptor molecules usually bind the corresponding ligands with high affinity. Therefore, it is not required that a receptor protein must be present in high concentration. Consequently, a thermoanalytic investigation employing calorimetry of ligand–receptor interactions in membrane systems appears not to be a very promising undertaking even under conditions where high sensitivity calorimeters are commercially

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available now. It is therefore understandable, that receptor binding studies generally are carried out with radioactively labeled ligands. Because high affinity binding is usually not only the result of electrostatic and hydrogen bond formation but also of hydrophobic interactions, many receptor ligands exhibit amphipatic properties. Therefore, such ligands are likely to bind not only selectively with high affinity to the corresponding receptor sites but also with low affinity to the lipid part of the membrane.

We will investigate here the interaction between a typical, steroid containing cardiac glycoside, Ouabain, with its receptor, Na,K-ATPase. This integral membrane protein acts as an ATP-driven alkali ion pump in all cells of higher organisms and maintains the cationic concentration gradients across plasma membranes. The specific inhibitory action of cardiac glycosides in the nanomolar concentration range was not only of importance in the course of the discovery of Na,K-ATPase [1,2], it also represents the therapeutic basis of the action of one of the oldest drugs, namely foxglove extracts, to treat congestive heart failure, described more than 200 years ago. The structure of Ouabain is shown in Fig. 1; some of its general properties as well as of similar compounds are described in [3–5]. The unsaturated lactone ring of the steroid moiety appears to be of importance for the molecular recognition. The

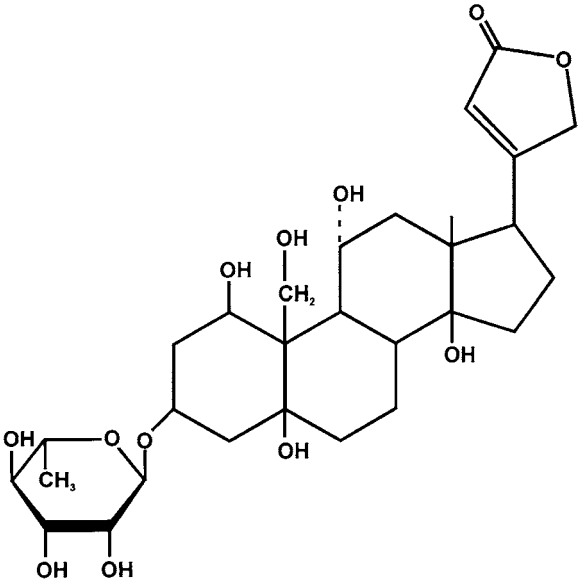

Fig. 1. Chemical structure of Ouabain.

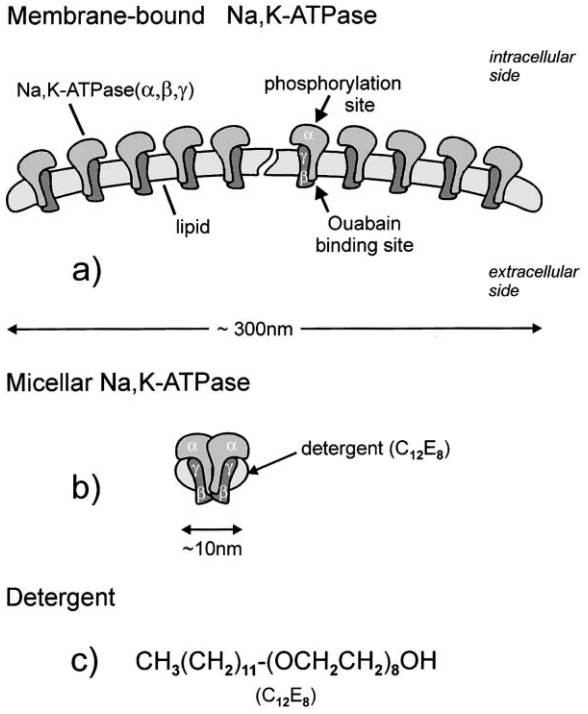

Fig. 2. Schematic illustration of (a) Na,K-ATPase in membrane fragments (purified membrane-bound preparation), (b) of micellar Na,K-ATPase (detergent solubilized preparation) and (c) of the structure of the detergent octaethyleneglycol monododecylether ($C_{12}E_8$).

binding of Ouabain is side-directed. This cardiac glycoside binds only to the former extracellular side of the protein (Fig. 2a). However, the ligand binding induces a functional response on the opposite membrane side of the enzyme, leading to the inhibition of ATP hydrolysis. The transduction of the response is thought to be transferred by a conformational transition. The observation, that cardiac glycoside binding is unusually slow [3–6], is consistent with this assumption. As a consequence of the very high affinity of Ouabain, its dissociation rate from the receptor site must be extremely slow. Experimental studies have confirmed this prediction [3–6]. Cardiac glycosides such as Ouabain are bound to Na,K-ATPase with high affinity only in the presence of millimolar concentrations of Mg^{2+} (type III complex). An even higher affinity, however, is observed upon phosphorylation of the enzyme, either with ATP in the presence of Na^+ (type I complex) or with inorganic phosphate (P_i),

which is denoted type II complex. K^+ exhibits an antagonistic effect and reduces strongly the affinity between Ouabain and its receptor site. Recently, considerable attention has been devoted to a surprising discovery in physiology, namely that Ouabain-type cardiotonic steroids are existing at least in mammals and appear to function as endogeneous Na,K-ATPase regulators [7–10]. The inhibition of Na,K-ATPase in the cellular system induces among other effects a diminished Ca^{2+} release from the cell by the Na^+/Ca^{2+}-exchanger, leading to a higher intracellular Ca^{2+} level which supports muscle contraction.

Na,K-ATPase is markedly expressed in specialized tissue such as of kidney or salt gland. In the cellular system, two K^+ are actively transported into and three Na^+ out of the cell, leading to the electrogenic transport of one Na^+. The enzyme is isolated from such tissues in form of membrane fragments with an average size around 300 nm (Fig. 2a). The purified fragments contain only this protein together with lipid molecules such as negatively charged phospholipids and neutral lipids, including cholesterol. The ordered orientation of the protein molecules is retained during the isolation. Thus, all ATP binding sites are exposed to the former intracellular side (Fig. 2a). Because integral membrane proteins are not water soluble, they can only be separated from their naturally occurring, local membrane surrounding by treatment with a suitable detergent. This treatment, which has to be carried out above the detergent's critical micelle concentration (CMC), leads to the formation of mixed protein–detergent micelles (Fig. 2b) and is generally called solubilization. A detergent that provides full retention of enzymatic Na,K-ATPase activity is octaethyleneglycol monododecylether ($C_{12}E_8$, Fig. 2c). It induces the formation of a protein dimer [11] in the mixed micelle (Fig. 2b).

Properties of Na,K-ATPase are reviewed in [12–14]. The enzyme originating from the tissues mentioned above has a molecular weight around 160 kDa. Its subunit composition consists of the catalytic major α-subunit, assumed to consist of 10 transmembrane segments, as well as of the β-glyco- and γ-protein, each containing only a single transmembrane segment. The investigation of mutated α-subunits has also led to putative details of the Ouabain binding site. It is assumed, for example, that three amino acid residues in the extracellular domain between transmembrane segment 1 and 2 are of importance for the interaction with cardiac glycosides [15].

2. Experimental

2.1. Chemicals

Ouabain (quality BioChemica), histidine, imidazole, tris-(hydroxymethyl)-aminomethane (Tris), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), *o*-phosphoric acid (quality MicroSelect), glycerol (quality MicroSelect) and trichloroacetic acid (quality puriss p.a.) were obtained from Fluka; HCl (quality Ultrapure), H_2SO_4, KCl, NaCl (quality Suprapur) and $MgCl_2$ (quality pro analysi) from Merck; sodium salt of ATP from Boehringer Mannheim; $C_{12}E_8$ from Nikkol Chemicals; $C_{12}E_{10}$ from Sigma. All other chemicals were of analytical grade, too.

2.2. Membrane-bound Na,K-ATPase

Na,K-ATPase was isolated from pig kidney and from dogfish (*Squalus acanthias)* rectal gland according to a modification of the procedure published in [16]. Concentrated stock solutions of the pig kidney enzyme were stored on ice in 25 mmol l^{-1} imidazole/HCl, 0.1 mmol l^{-1} DTT pH 7.5, and of the dogfish enzyme in 30 mmol l^{-1} histidine/HCl, 0.1 mmol l^{-1} EDTA pH 6.8 containing 25% glycerol (w/w). Protein concentrations were determined according to the methods of Lowry et al. [17] and Popov [18]. Amino acid determinations were carried out by the courtesy of Dr. M. Raida. For the pig kidney enzyme, the protein content according to Popov, which was identical to the value obtained from the amount of non-hydrolizable amino acids (related to the α1β1 sequences), was 24% lower than the value according to Lowry. The higher Lowry value is attributed to the interference with phospholipids. In the case of the dogfish enzyme, the protein content according to Popov was 27% lower than the Lowry value and the amino acid determination provided a 5% lower value (related to the Torpedo α and β sequences because the dogfish sequences are not yet known) than the Popov determination. The cholesterol content, determined according to [19,20], was 0.35 mg mg^{-1} protein (Popov) for the pig kidney and 0.4 mg mg^{-1} protein

(Popov) for the dogfish enzyme. The phospholipid content of the dogfish enzyme, determined according to [21], was around 2.3 μmol phosphate mg^{-1} protein (Popov). Assuming a phospholipid molecular weight of 755 Da, this corresponds to 1.75 mg phospholipid mg^{-1} protein. The enzymatic activity was determined in the presence of excess substrate, 3 mmol l^{-1} ATP, at 37°C in 30 mmol l^{-1} histidine/HCl, 130 mmol l^{-1} NaCl, 20 mmol l^{-1} KCl and 3 mmol l^{-1} $MgCl_2$ pH 7.5. The reaction was stopped at different time intervals with trichloroacetic acid and the amount of hydrolyzed P_i was determined according to [22]. The specific activities ranged between 32 and 36 μmol P_i min^{-1} mg^{-1} protein (Popov). The enzymatic activity in the presence of 1 mmol l^{-1} Ouabain was less than 1%. The molecular weight for the pig kidney enzyme ($\alpha\beta\gamma$) is assumed to be 158 kDa; that of the dogfish enzyme 165 kDa.

2.3. Micellar Na,K-ATPase

Purified dogfish Na,K-ATPase in 30 mmol l^{-1} histidine/HCl, 0.1 mmol l^{-1} EDTA pH 6.8 containing 25% glycerol (w/w) was solubilized similar to [23] with $C_{12}E_8$ under gentle stirring in ice. The molar ratio between detergent and protein was 725:1. After centrifugation at 280,000 × *g* and 8°C as well as a subsequent Ultrafree-MC filtration (0.1 μm; Millipore), the sample was separated with 30 mmol l^{-1} histidine/HCl, 0.05 mmol l^{-1} EDTA pH 7.0 containing 0.1 mg $C_{12}E_8$ ml^{-1} on a detergent saturated Sephacryl S-300 (Pharmacia Biotech) column. The eluted main fraction was used for the analytic and calorimetric measurements. The protein content was determined as described before. The contents of cholesterol and phospholipid were around 0.15 mg mg^{-1} protein (Popov) and 0.7 μmol phosphate mg^{-1} protein (Popov), respectively. The molecular weight is assumed to be 150 kDa because the γ-subunit dissociates in the presence of the detergent [24]. The enzymatic activity at 37°C, determined with 2–5 μg protein ml^{-1}, was determined as mentioned above, but in the presence of 70 μg $C_{12}E_{10}$ ml^{-1}. ATP hydrolysis was stopped upon addition of SDS leading to a final concentration of 1% (w/w). The specific activity was identical to that of the membrane-bound enzyme. Further details concerning preparations and analytical characterizations are described in [25].

2.4. Ouabain inhibition

Enzymatic activities were determined in the medium specified above at 25 ± 0.5°C in the presence of different Ouabain concentrations (about 3×10^{-8} to 10^{-4} mol l^{-1}). Before substrate addition in form of a concentrated, pH adjusted ATP solution, the enzyme was incubated for a duration of 30 min in the presence of the chosen Ouabain concentration to provide sufficient time for binding. The evaluation was done with the program Origin (MicroCal) on the basis of a 1:1 complex formation model.

2.5. Titration calorimetry

The membrane-bound enzymes were centrifuged at 280,000 × *g* and 4°C and the pellets were resuspended in the corresponding titration buffer. Micellar Na,K-ATPase was applied immediately after its preparation. The protein concentrations were determined as given above. The calorimetric titrations were performed with the MCS ITC setup (MicroCal) at 25 ± 0.15°C. The enzyme solutions were thoroughly degassed (vacuum) and then transferred to the calorimetric cell (volume 1.353 ml). After temperature equilibration, a 100 μl syringe equipped with a stirring paddle and filled with the degassed titrant solution was installed. After temperature re-equilibration, the titration was started. The additions under continuous stirring were controlled by a PC. Due to the very slow binding of Ouabain, an unusually long time between titrant additions (420 s) had to be chosen. The evaluations of the titrations were carried out with the manufacturer's software.

2.6. Solutions

A concentrated solution of Tris/P_i was prepared by neutralizing phosphoric acid with Tris. Unless KCl was added, all solutions were nominally free of K^+ by avoiding electrolyte contaminations from the combined pH electrode.

3. Results and discussion

3.1. Inhibition studies

In order to obtain information about the affinity between Ouabain and membrane-bound pig kidney as

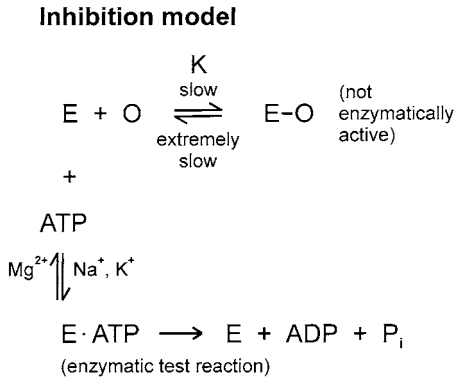

Fig. 3. Reaction model: enzymatic activity determination for the characterization of the inhibition of Na,K-ATPase (E) due to Ouabain (O) binding (*K*: inhibitor binding constant).

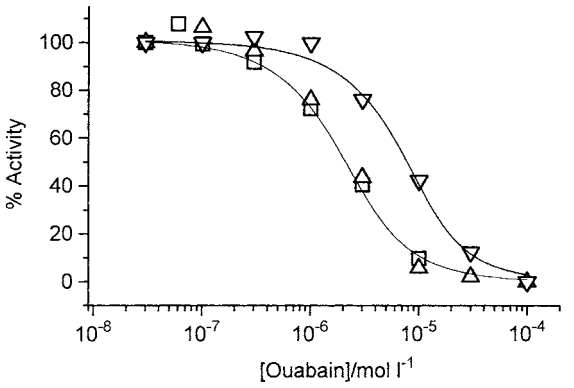

Fig. 4. Ouabain inhibition of the enzymatic activity of Na,K-ATPase at pH 7.5 and 25°C: semilogarithmic plot of % activity as a function of Ouabain concentration for the purified, membrane-bound pig kidney (▽) and dogfish (△) as well as for the purified, micellar dogfish enzyme (□). The ionic composition of the medium is given in Section 2. The solid lines correspond to fits related to a 1:1 binding model (cf. reaction model in Fig. 3).

well as membrane-bound and micellar dogfish Na,K-ATPase, inhibition studies are carried out by investigating the enzymatic ATP hydrolysis as a function of Ouabain concentration. Details of the experimental conditions are described in Section 2. Comparatively long Ouabain incubation times must be chosen to account for the exceptionally slow binding kinetics. As a consequence of this observation, the displacement of the pre-adjusted equilibrium between the enzyme and Ouabain as a consequence of the addition of the substrate ATP (cf. Fig. 3) can be neglected for the duration of the enzymatic test measurement. The evaluation of the experimental inhibition data is based on a model related to 1:1 complex formation. The results are illustrated in Fig. 4. The membrane-bound pig kidney enzyme, which consists of the α1 isoform, is characterized by a inhibition constant of 8.5×10^{-6} mol l^{-1}. Under our conditions, this corresponds to a stability or affinity constant K of 1.25×10^{5} mol^{-1} l (reciprocal of the inhibition constant). This value appears not to be very high but this is the consequence of the reduction of the interaction between Ouabain and its receptor site in the presence of high concentrations of K^+. The membrane-bound dogfish enzyme, which predominantly consists of the α3 isoform [24], exhibits an about 3.5 times stronger interaction with Ouabain and is characterized by an inhibition constant of 2.2×10^{-6} mol l^{-1}, respectively, a stability constant of 4.5×10^{5} mol^{-1} l. For the micellar dogfish enzyme, solubilized with the detergent $C_{12}E_8$ (described in Section 2), the same affinity is observed as for the membrane-bound preparation. This is considered as an indication that the receptor binding site of the enzyme is not altered upon the transition of Na,K-ATPase from the membrane-bound to the micellar state as well as during the separation on Sephacryl. Although the pig kidney enzyme can also be solubilized under the same conditions, full and stable enzymatic activity cannot be achieved as in the case of the dogfish preparation and the remaining enzymatic activity decays quickly.

3.2. Calorimetry

Because of the high Ouabain binding affinity, calorimetric titrations of Na,K-ATPase have to be carried out under conditions of very low enzyme concentration. In Fig. 5, a typical titration of membrane-bound pig kidney enzyme in 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.0 containing 25% glycerol (w/w) is shown. Glycerol is added to prevent aggregation between membrane fragments, which is even more important in the case of the dogfish enzyme. The intense signals result upon single ligand additions and are characteristic of an exothermic binding process. The intensity varies only slightly in the course of the titration. However, characteristic changes of the half-line widths of the heat signals are observed. The signal

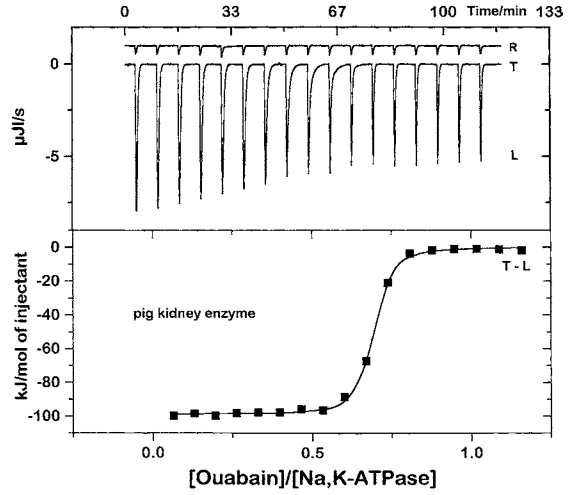

Fig. 5. (Top) Calorimetric titration of 20 µmol l^{-1} purified pig kidney Na,K-ATPase in membrane fragments at 25°C in 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.0 containing 25% glycerol (w/w) with 0.25 mmol l^{-1} Ouabain (17 × 7 µl) in the same medium (T). The reference titration (plot is shifted by an offset) is carried out analogously but in the absence of the enzyme (R). (Bottom) Plot of the determined titration enthalpy of the selective ligand binding in dependence of the concentration ratio (details are given in the text, the parameters related to the theoretical curve — solid line — are listed in Table 1).

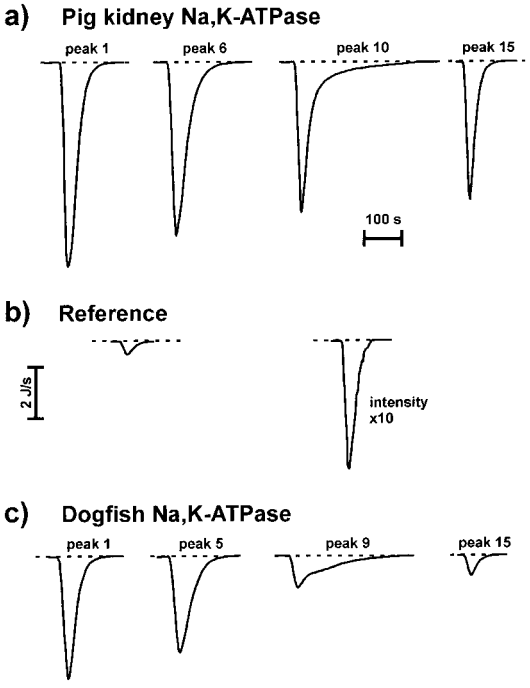

Fig. 6. Comparison of characteristic heat signals observed during the titrations shown in Figs. 5 and 7 (details are given in the corresponding figure legends).

width is larger at the beginning than at the end of the titration and reaches a maximum in an intermediate stage. Large half-line widths can be attributed to very slow Ouabain binding kinetics and imply that the heat evolvement is slower than the response of the heat detection system of the setup. At the beginning of the titration, Ouabain binding is still comparatively fast although the width of the first peak (Fig. 6a) is already larger than that observed during the reference titration (Fig. 6). With increasing number of Ouabain additions, the peaks become broader and broader until they are narrow again from peak 11 on (for example peak 15 in Fig. 6a). In addition, the peak intensity remains constant from peak 11 on, although the intensities are much higher than in the case of the reference titration (R in Fig. 5 top). The constant signals of the reference titration are mainly attributed to the heat of Ouabain dilution. On the basis of these considerations, we assign the second half of the Na,K-ATPase titration with Ouabain (Fig. 5) to an exothermic, low affinity binding process giving rise to the same heat change upon every Ouabain addition. In molecular terms, this low affinity binding process can be attributed to non-specific Ouabain binding to the lipid regions of the membrane fragments. Consequently, the first part of the titration (until peak 10 in Fig. 5) is due to heat changes consisting of the sum of the low affinity and the high affinity binding signal. The exothermic, high affinity binding signal characterizes the interaction of Ouabain with its specific receptor site. Whereas the low affinity signals remain constant during the titration, the heat change attributed to specific binding evolves slower and slower. With progressing titration, the width of the receptor binding signal increases steadily (cf. peaks 6 and 10 in Fig. 6a) because the concentration of unloaded receptor site decreases. Thus, the binding rate, which corresponds to the product of the rate constant times the product of the free Ouabain and receptor site concentrations, decreases after every addition until saturation of the site is reached.

For the quantitative evaluation of the titration T in Fig. 5 (top), the arithmetic mean of the integrated signals 12–17 (L) is subtracted from the measured heat

Table 1
Thermodynamic parameters of Ouabain binding to purified membrane-bound and micellar Na,K-ATPase at 25°C in different media[a]

Source of enzyme	Medium[b]	Concentration (μmol l^{-1})	K (mol^{-1} l)	n	ΔH (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
Pig kidney (membrane-bound)	A	4.0	$(3.0 \pm 1.5) \times 10^7$	0.55 ± 0.04	-82 ± 4	-132 ± 9
	A	20	(5×10^7)[c]	0.66 ± 0.04	-92 ± 5	(-160)[c]
	B	14	(10^8)[c]	0.65 ± 0.05	-75 ± 4	(-100)[c]
Dogfish (membrane-bound)	A	1.7	$(5.5 \pm 2.5) \times 10^7$	0.54 ± 0.05	-97 ± 5	-175 ± 15
	A	3.8	$(1.5 \pm 1.0) \times 10^8$	0.55 ± 0.05	-95 ± 5	-160 ± 20
	A	19	(10^8)[c]	0.60 ± 0.04	-96 ± 5	(-165)[c]
	B	13	(10^8)[c]	0.62 ± 0.05	-85 ± 5	(-130)[c]
Dogfish (micellar)	C	3.4	$(2 \pm 1) \times 10^8$	0.63 ± 0.06	-88 ± 6	-135 ± 15

[a] K: stability constant, n: stoichiometric coefficient.
[b] Media: A — 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.0 containing 25% glycerol (w/w); B — 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.5; C — 10 mmol l^{-1} imidazole/HCl, 0.05 mmol l^{-1} EDTA, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i, 0.1 mg $C_{12}E_8$ ml^{-1} pH 7.0.
[c] Approximate value because of reduced accuracy due to high enzyme concentration.

changes. This leads to the concentration ratio dependence of the binding heat changes shown in Fig. 5 (bottom, T–L). The resulting thermodynamic parameters are given in Table 1. The binding constant and subsequently also the entropy change cannot be determined with high precision under the chosen conditions for this figure because the receptor concentration of 20 μmol l^{-1} is too high. However, a reasonable determination of the enthalpy change and of the stoichiometric coefficient is possible. Evidently, saturation of the receptor's Ouabain binding site occurs at a molar ratio of about 0.66 (Fig. 5 bottom), which is significantly smaller than one. From the corresponding values in Table 1, it is concluded that the binding of Ouabain is determined by enthalpic interactions. The large enthalpy contribution of more than -80 kJ mol^{-1} is counterbalanced to a large extent by the high, negative entropy change (cf. Table 1). A more precise quantitative determination of all thermodynamic parameters is possible with a titration with a reduced enzyme concentration of 4.0 μmol l^{-1}. The experiment leads to a binding constant of 3×10^7 mol^{-1} l and a stoichiometric coefficient of 0.55 (Table 1). The presence of glycerol in the titration medium appears to affect the values of the thermodynamic parameters, too. In the absence of glycerol, the absolute values of ΔH and ΔS are slightly smaller (Table 1).

In Fig. 7, a corresponding calorimetric titration with the membrane-bound dogfish enzyme (19 μmol l^{-1}) in the presence of the glycerol containing medium is shown. This titration looks now much more similar to a standard calorimetric titration profile. Again, comparatively broad heat signals are observed, characterized by half-line widths that increase markedly towards saturation of the specific receptor site (cf.

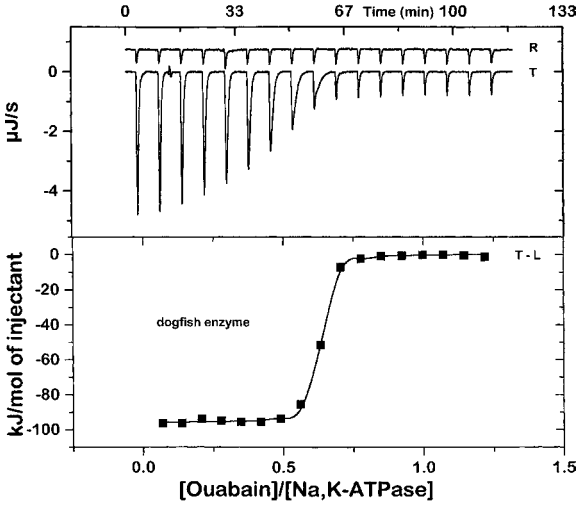

Fig. 7. (Top) Calorimetric titration of 19 μmol l^{-1} purified dogfish Na,K-ATPase in membrane fragments at 25°C in 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.0 containing 25% glycerol (w/w) with 0.25 mmol l^{-1} Ouabain (17×7 µl) in the same medium (T). The reference titration (plot is shifted by an offset) is carried out analogously but in the absence of the enzyme (R). (Bottom) Plot of the determined titration enthalpy of selective ligand binding in dependence of the concentration ratio (details are given in the text, the parameters related to the theoretical curve — solid line — are listed in Table 1).

Fig. 6c). After the 9th Ouabain addition, the signal is again very broad. From the 10th peak on, narrow heat signals of identical intensity are detected. These latter signals are also more intense than those of the corresponding reference titration (R in Fig. 7 top), but clearly much less intense than the corresponding signals of the pig kidney membrane fragment titration (T in Fig. 5). This difference related to the low affinity Ouabain binding is attributed to a different lipid composition of the dogfish salt gland membrane fragment. Either the affinity of nonspecific Ouabain binding is reduced and/or the enthalpy change is smaller than in the case of the pig kidney membrane. Again for the purpose of a quantitative evaluation of the titration T in Fig. 7 (top), the arithmetic mean of the integrated signals 11–17 is subtracted from the measured heat changes, leading to the dependence shown in Fig. 7 (bottom, T–L). Also here, saturation of the Ouabain binding site is reached considerably before one equivalent of ligand is added. The resulting thermodynamic parameters are also given in Table 1. Higher precision for the binding constant is obtained from titrations performed with much lower receptor concentrations (Table 1). The evaluation of a titration with only 1.7 μmol l^{-1} dogfish enzyme is illustrated in Fig. 8 and leads to a binding constant of 5.5×10^7 mol^{-1} l. The Ouabain binding constant is higher for the dogfish protein than for the pig kidney enzyme, which is consistent with the results of our inhibition studies (cf. Fig. 4). Also the absolute ΔH and ΔS values appear to be higher (Table 1). Omitting glycerol leads also in the case of the dogfish enzyme to somehow smaller enthalpy and entropy changes.

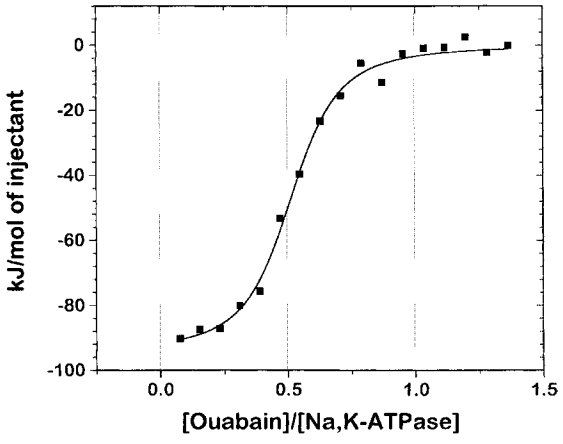

Fig. 8. Evaluation of the calorimetric titration of 1.7 μmol l^{-1} purified dogfish Na,K-ATPase in membrane fragments at 25°C in 10 mmol l^{-1} imidazole/HCl, 3 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Tris/P_i pH 7.0 containing 25% glycerol (w/w) with 0.05 mmol l^{-1} Ouabain (17×7 μl) in the same medium: plot of determined titration enthalpy of selective ligand binding as a function of the concentration ratio (details are given in the text, the parameters related to the theoretical curve — solid line — are listed in Table 1).

The origin of the observed low value of the stoichiometric coefficient (smaller than 1) is not known and could, for example, be the consequence of membrane particle aggregation or of a bound natural ligand, already present prior to enzyme isolation. In order to check such possibilities as well as the lipid binding hypothesis mentioned above, micellar Na,K-ATPase is prepared by solubilizing the dogfish membrane fragments with $C_{12}E_8$ and performing a subsequent Sephacryl chromatography to obtain a mixed protein/detergent micelle. This mixed micelle preferentially contains the diprotomer [11] in the presence of only about one-third to one-forth of the original lipid content (details are given in Section 2). Again, calorimetric titrations are carried out with Ouabain in the presence of the detergent above its CMC, however in the absence of glycerol. Essentially the same results are obtained as for the membrane-bound preparation (cf. Table 1). This suggests that the properties of Na, K-ATPase with regard to cardiotonic steroid binding have not been altered as a consequence of the transition from the membrane-bound to the micellar phase. In addition, we conclude that there is no evidence for membrane particle aggregation which could have affected the value of the stoichiometric coefficient. However, the heat signal observed upon Ouabain addition after receptor saturation, characteristic of nonspecific low affinity binding, is smaller than for the membrane-bound state which correlates, as expected, with the decrease of the lipid content, which is consistent with our hypothesis.

The observed, unusually slow decrease of the heat signal due to specific Ouabain binding (e.g. peak 6 of the pig kidney and peak 9 of the dogfish enzyme in Fig. 6) allows a coarse estimation of the binding rate constant. Because the single ligand addition induces only comparatively small concentration changes, we can consider every titration step as a chemical relaxation experiment, where the perturbation of the equilibrium system is fast compared to its re-equilibration

[26]. Thus, we can apply the relaxation expression for a one-step binding process [26]:

$$\tau^{-1} = k_{on}([\text{receptor}]_{eq} + [\text{ligand}]_{eq}) + k_{off}$$

where τ^{-1} is the relaxation time of the chemical system and the symbol $[\ ]_{eq}$ represents the equilibrium concentration. For this estimation, k_{off} can be neglected because its value is much smaller than $1\ s^{-1}$. Thus, on the basis of a coarse estimation of the relaxation time and the resulting equilibrium concentrations, a k_{on} value of the order of $10^4\ mol^{-1}\ l\ s^{-1}$ is obtained, which is consistent with the results of kinetic studies employing a fluorescent derivative of Ouabain [6,25]. Such a low value for a binding rate constant implies, that at least a two-step binding model has to be considered, as shown in Fig. 9. The formation of a low affinity Ouabain/receptor complex precedes a slow conformational rearrangement leading to the final high affinity complex. If we make the plausible assumption, that the dissociation rate constant k_{21} of the intermediate complex is much higher than the rate constant k_{23} of the conformational transition leading to the final state, the determined k_{on} has then the meaning of the product of the equilibrium constant of the low affinity complex (K_1) times the rate constant k_{23} (Fig. 9). The value of this product term can be much smaller than what would be expected for a simple one-step binding process. In addition, under these conditions k_{off} corresponds to the dissociation rate constant k_{32}. Thus, the suggested interpretation concerning a rate-limiting protein conformational transition as a consequence of initial low affinity Ouabain binding is in good agreement with the experimentally obtained results.

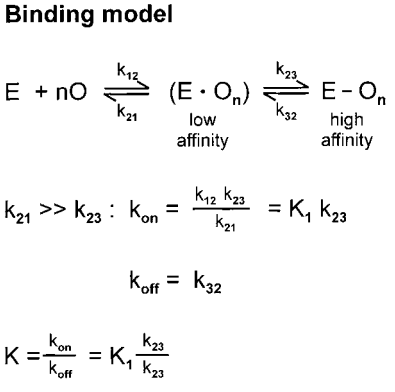

Fig. 9. Determination of binding rate constant from calorimetric titration data: reaction model for the evaluation of the time dependence of the heat evolved upon Ouabain (O) binding to Na, K-ATPase (E). Details are given in the text (*n*: stoichiometric coefficient).

4. Conclusions

This investigation demonstrates the advantage of the applied calorimetric technique which allows a simple and straightforward differentiation between nonspecific lipid and specific receptor binding of the ligand together with a determination of the stoichiometric coefficient. Even binding constants as high as $5 \times 10^7\ mol^{-1}\ l$ are determined with good precision as long as the enthalpy change is comparatively large. Taking into consideration that the concentration of receptor binding site is about 0.6 times lower than that of the protein, the lowest applied concentration for a titration with the dogfish enzyme was only about 1 μM.

A stoichiometric coefficient around 0.60 implies that about two-fifth of the Na,K-ATPase protomers, independent whether membrane-bound or in the purified micellar phase, do not bind Ouabain, at least not strongly. Recent phosphorylation studies of pig kidney and dogfish enzyme [24,27] indicate, that only 2.5–2.7 nmol P_i are covalently bound per mg of enzyme (Lowry determination). If we consider that the Popov value is markedly lower (cf. Section 2), we obtain a value for the stoichiometric coefficient of ATP-induced phosphorylation which is nearly identical with the result of our Ouabain studies. Since the errors of such protein determinations are estimated to be up to 10%, we conclude that about half of the enzyme molecules, consisting of the subunits $\alpha\beta\gamma$, are not in a functional state. Because the purified dogfish enzyme is so much more stable than the corresponding pig kidney preparation, we consider it as unlikely that this is the result of partial denaturation during the isolation procedure. It is therefore possible, that the enzyme of our preparation functions as a dimer (diprotomer) consisting only of a single high affinity binding site, as suggested earlier ([28] and citations therein), or that the function of about one-half of the enzyme molecules is blocked, possibly due to a currently unknown interaction. On the basis of these findings, the enzymatic activity per mg of protein, related only to the

active part of the preparation, has to be expected to be twice as large as given before in the Section 2, namely above 60 μmol $P_i\,min^{-1}\,mg^{-1}$ protein. This is not unresonable because a fully active nasal Na,K-ATPase, isolated from salt-stressed ducks, has recently been reported which indeed exhibits such a high activity and also a phosphorylation stoichiometric coefficient around 1.0 [29,30].

The high affinity and large negative enthalpy change found here for Ouabain binding can be considered as typical for multiple interactions between the ligand and the receptor site, such as involving the formation of several hydrogen bonds. The unfavorable, large negative entropy change upon binding is not characteristic of a substancial solvate liberation upon the binding of Ouabain to a solvent accessible binding pocket of the protein. It would, for example, be consistent with an increase in solvate interaction as a consequence of a conformational transition leading to a an increased solvent accessibility of the protein. The breaking of a salt bridge and its exposure to the solvent could contribute in such a direction, although this represents a speculative interpretation. Evidently, the given interpretation of the estimated binding kinetics is consistent with the concept of a substancial conformational rearrangement. Such a molecular concept would be meaningful also from the biochemical point of the view, because the binding of the receptor Ouabain to the extracellular side of the protein leads to the inhibition of the functional enzymatic activity at the opposite, intracellular side of the membrane. Future structural studies will be required to reach decisions about such detailed molecular aspects.

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