Journal of Thermal Analysis, Vol. 48 (1997) 437-445

MEMBRANE-BOUND Na^+,K^+-ATPase AS THE CARDIAC GLYCOSIDE RECEPTOR
A thermochemical characterization

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Abstract

DSC studies are carried out to characterize Na^+,K^+-ATPase isolated from pig kidney in its natural membrane environment as well as in its purified state upon detergent treatment. The transition temperatures of the investigated thermal protein unfolding process, observed between 43 and 64.5°C, depend on the local membrane environment as well as on *pH*. In addition, the transition temperature is significantly increased upon binding of different cations and ligands which are known to interact with the enzyme. Evidence for a lipid phase transition around 23°C in the original biological membrane is reported.

The application of a calorimeter equipped with removable cells appears to be more suitable for the investigation of this type of membrane sample than an instrument with fixed capillary cells. Filling the sample capillary cell with an usual syringe, consisting of a long and thin needle, can influence the experimental results.

Na^+,K^+-ATPase acts as the receptor for cardiac glycoside binding. The thermodynamic parameters of this binding process are determined by titration calorimetry. The binding of ouabain, as a typical example, is unusually slow and is enthalpy driven. The enthalpy change upon binding enthalpy is -75 kJ mol^{-1} at 25°C. The surprisingly low stoichiometric coefficient, resulting from an evaluation based on a simple one step binding model, is interpreted in terms of a dimeric receptor unit.

Keywords: cardiac glycoside binding, DSC, membrane protein, membrane receptor binding, Na^+,K^+-ATPase, thermal unfolding, titration calorimetry

Introduction

The ion pump Na^+,K^+-ATPase is an integral membrane enzyme that actively transports Na^+ and K^+ against the respective cellular concentration differences by converting the resulting free energy of hydrolysis of adenosine triphosphate (ATP) to the corresponding energy consuming, active transport processes [1]. Because of their hydrophobic nature, integral membrane proteins are not water soluble; they can only be isolated in form of membrane fragments or of mixed detergent micelles. In the detergent solubilized state the hydrophobic regions, originally in contact with the lipid molecules of the natural environment, are assumed to be covered by detergent molecules. Based on general considerations, it is assumed that the properties

0368–4466/97/ $ 5.00
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of an integral membrane protein do not only depend on the particular protein itself but also on the properties of the surrounding lipid environment. Purified Na^+,K^+-ATPase used in this study is isolated in form of slightly curved discs with retention of the original, ordered orientation of the protein molecules, as indicated schematically in Fig. 1. These sheets have a diameter of about 280 nm [2, 9].

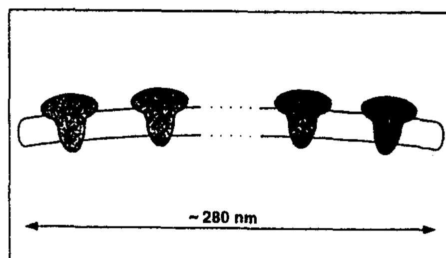

Fig. 1 Schematic illustration of the arrangement of oriented Na^+,K^+-ATPase molecules in the disk shaped membrane sheet

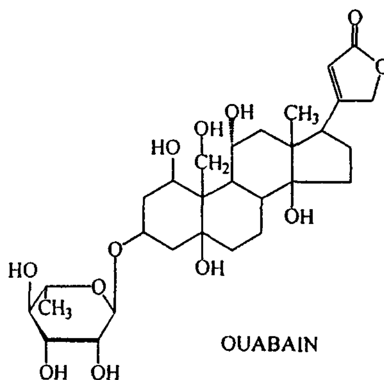

Fig. 2 Chemical structure of ouabain

The enzyme consists of two peptides, the enzymatically active α unit with a molecular weight around 100 kD and the ß unit with a molecular weight around 50 kD. With regard to pharmacological aspects, Na^+,K^+-ATPase is considered to act as the cardiac glycoside receptor [3]. Except for the rat kidney enzyme, for example, most renal enzymes are specifically inhibited by cardiac glycosides such as ouabain (Fig. 2) in the nanomolar concentration range. The affinity depends on the composition of the aqueous medium. This inhibition can be correlated with the inotropic effect of these drugs on heart muscle. An interesting aspect concerning membrane sidedness is due to the fact that cardiac glycosides are only bound to the extracellular membrane side of the original cellular system.

In order to obtain information about the thermal stability of the enzyme in media of different composition thermochemical properties of crude and purified Na^+,K^+-ATPase are investigated by differential scanning calorimetry (DSC). The thermodynamic parameters of the binding of ouabain, a typical cardiac glycoside repre-

sentative, to Na^+,K^+-ATPase as its receptor are determined by titration calorimetry.

Experimental

Enzyme preparation

Crude Na^+,K^+-ATPase is prepared from pig kidney red outer medulla as microsomal fraction by carrying out different homogenization and centrifugation steps at *pH* 7.2 according to [4], leading to an ATPase activity at 37°C around 0.6 μmol phosphate min^{-1} mg $protein^{-1}$. In this state, most of the catalytic ATP sites are not accessible to the substrate. Purified Na^+,K^+-ATPase is obtained after detergent activation with sodium dodecyl sulfate and density gradient centrifugation, fractionation and washing steps [5, 6] with an ATPase activity of 35 μmol phosphate min^{-1} mg $protein^{-1}$ at 37°C. Protein determinations are done according to Lowry *et al.* [7] by employing a modified procedure. Imidazole used as buffer compound is obtained from Fluka as Micro Select for luminescense spectroscopy grade and has been sublimed to minimize the trace cation content. The buffers used in this study have not been in contact with a *pH* electrode to avoid extra KCl contamination. Na^+,K^+-ATPase concentrations are calculated on the basis of an assumed molecular weight of 150 kD. Abbreviations: ATP, adenosine 5'-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Calorimetry

DSC studies are performed employing the Setaram MicroDSC II consisting of removable DSC cells and the MicroCal DSC instrument equipped with fixed capillary cells. Isothermal titrations at 25°C are carried out with a MicroCal isothermal titration calorimeter. The inner diameter of the filling syringe needle of the Microcal DSC instrument is considerably smaller than that of the Microcal titration calorimeter.

Results and discussion

Thermal unfolding

In order to characterize the stability and the general features of Na^+,K^+-ATPase at different stages of the isolation a differential scanning microcalorimetry (DSC) study by employing the Setaram DSC instrument has been carried out. Figure 3 shows two successive DSC runs of the crude Na^+,K^+-ATPase preparation consisting of the microsomal fraction at *pH* 6.5. Besides some comparatively broad endothermic transitions observed during the first run around 23, 47 and 72°C, a fairly narrow endothermic transition with a half width of about 3°C is detected at a temperature of 57.5°C. The latter transition, which is almost irreversible (cf. second run in Fig. 3), is attributed to Na^+,K^+-ATPase. Up to this stage of the preparation, no detergent has been applied. It appears that calorimetry offers a comparatively

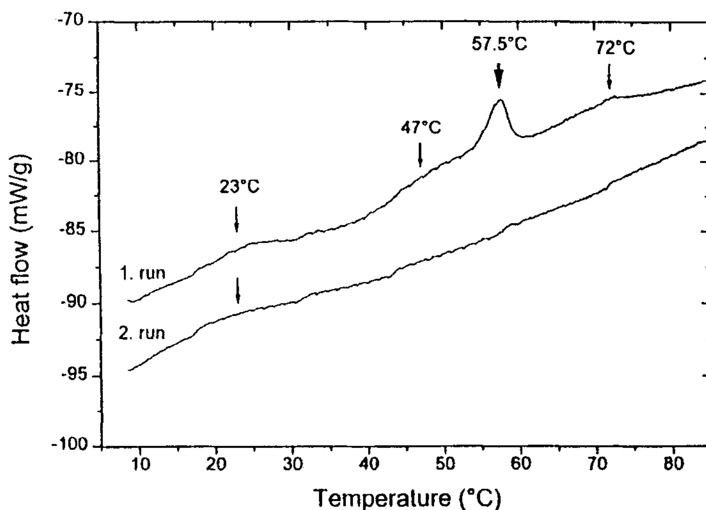

Fig. 3 DSC experiment of the microsomal fraction (11.6 mg protein) in 25 mM imidazole/HCl *pH* 6.5 ranging from 5 to 85°C (temperature scan 0.3°C min^{-1}). Details are given in the text

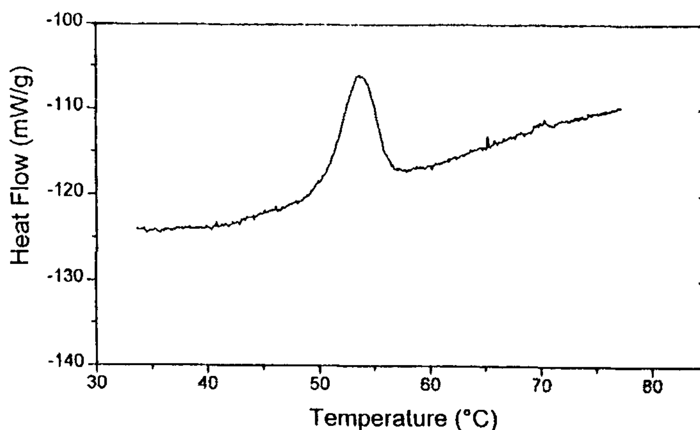

Fig. 4 DSC experiment of purified Na^+,K^+-ATPase (3.73 mg) in 25 mM imidazole/HCl *pH* 6.5 ranging from 5 to 80°C (temperature scan 0.3°C min^{-1}). The transition temperature is 53.7°C; details are given in the text

simple possibility to estimate the Na^+,K^+-ATPase content in such a crude enzyme preparation. The reversible transition in Fig. 3 observed at 23°C is attributed to a lipid phase transition.

A typical DSC run of purified Na^+,K^+-ATPase in the same buffer, obtained again with the Setaram instrument, is characterized now by a single endothermic transition with a transition temperature of 53.7°C, as shown in Fig. 4. The endothermic enthalpy change of the unfolding transition is around 1.5 MJ mol^{-1} and the

half width of the transition is about 3.5°C. Also the transition of the pure enzyme is almost irreversible. Based on the assumption that the transition enthalpy of the enzyme is the same in the crude microsomal preparation, we can now estimate from the area of the 57.5°C transition in Fig. 3 the amount of Na^+,K^+-ATPase present in the microsomal fraction which turns out to be around 20% of the total amount of protein. This correlates well with the yield of the preparative isolation of the purified enzyme from the microsomal fraction. If the transition temperatures of the crude and of the purified enzyme are compared, it is surprising to note that the enzyme in its purified but detergent treated state exhibits a lower transition temperature than in the crude microsomal preparation, although the half widths of the transitions are similar. This could imply that the surroundings of the protein have been changed to some extent upon detergent treatment during the purification but the overall protein conformation or its degree of order has remained, as concluded from the similarity of the half widths of the transitions in both states of the preparation.

According to protein denaturation studies published by Privalov and Gill [8] a value around 1.1 kJ mol^{-1} per amino acid can be expected. Therefore, for Na^+,K^+-ATPase a denaturation enthalpy change of the order of magnitude of −1.1 MJ mol^{-1} could thus be expected. Because the experimentally determined transition enthalpy change for Na^+,K^+-ATPase is close to the expected value we assume that a global denaturation of the enzyme and not only a local unfolding process has been investigated. Less enzymatically active preparations of the enzyme are characterized by considerably broader transitions, however, only by slightly lower transition temperatures. It is therefore concluded that the half width of the thermal denaturation transition can be applied as a criterium to estimate the purity of the preparation.

Unexpected problems arose in nearly all attempts to measure the thermal denaturation of Na^+,K^+-ATPase in the MicroCal DSC instrument. Instead of or in addition to the expected endothermic transition an exothermic transition with a transition temperature around 48°C was observed in the same medium. This exothermic process could be indicative of a dominating association process which can exceed the expected endothermic denaturation process. Also this unexpected exothermic transition is irreversible. The MicroCal instrument is equipped with fixed capillary cells which have to be filled with a syringe equipped with a long capillary needle, whereas the removable cells of the Setaram instrument are filled with a 1 ml standard Eppendorf pipette. The exothermic process observed with the MicroCal instrument is attributed to a modification of the properties of the Na^+,K^+-ATPase preparation as a consequence of the streaming of the enzyme solution through the filling capillary of the syringe under pressure which is assumed to lead to shearing forces. This explanation was confirmed by a control experiment where the removable Setaram cell was filled with the syringe of the MicroCal DSC instrument. Also under these circumstances an exothermic process was observed with the Setaram DSC instrument. This result clearly indicates that filling techniques can strongly influence the results obtained with biological samples. In order to test the effect of shearing forces on the membrane particle size of the preparation, Na^+,K^+-ATPase samples were extruded through 100 nm filters [9]. Electron microscopy

and dynamic light scattering studies indicated a reduction of particle size upon extrusion [9]. The enzymatic activity, however, remained.

Because the thermal denaturation temperature can be used as a sensitive measure for the characterization of the overall thermal stability of the protein, DSC studies employing the Setaram instrument have been carried out on Na^+,K^+-ATPase at different *pH* values as well as at a constant *pH* of 7.5 in the presence of different ligands known to interact with this enzyme. Around *pH* 8.0 the denaturation temperature is observed at about 57°C, which is similar to that of the enzyme in the microsomal fraction. Decreasing the *pH* leads to a reduction of the transition temperatures. At *pH* 5.0 the transition temperature is already close to 43°C. From the dependence of the transition temperature on *pH* it is concluded that at least one acidic group with a *pK* value around 6.0 is likely to influence the structure of the protein significantly. Addition of 6 mM KCl to Na^+,K^+-ATPase in 25 mM imidazole/HCl at *pH* 7.5 leads to an increase of the transition temperature from 56 to 59.5°C. Similarly, after the addition of 3 mM $MgCl_2$ to the enzyme in 25 mM imidazole/HCl *pH* 7.5, a 2.5°C increase of the transition temperature is observed. If 3 mM Tris phosphate is added to the $MgCl_2$ containing solution, which leads to the phosphorylation of the enzyme, a further increase by about 1°C is found. The presence of millimolar concentrations of Mg^{2+} as well as of phosphate represents a requirement to achieve high affinity cardiac glycoside binding. Upon addition of 0.1 mM ouabain to the previous medium consisting of the phosphorylated enzyme, a transition temperature of 64.5°C is observed which represents a 5°C increase compared to that of the phosphorylated state. These results obtained in the presence of different cations and ligands indicate that specific binding processes can lead to marked stabilizations of the Na^+,K^+-ATPase structure, which to some extent is consistent with earlier studies [10] and with time dependent inactivation studies based on enzymatic activity measurements [10, 11].

Isothermal titrations

In order to get information about the thermodynamic parameters of cardiac glycoside binding titrations have been carried out at 25°C with 0.5 mM ouabain in 10 mM imidazole/HCl containing 3 mM $MgCl_2$ and 3 mM phosphate *pH* 7.5 and the purified pig kidney enzyme in the same medium by employing the isothermal MicroCal titration calorimeter (Fig. 5A). The measured heat changes observed upon subsequent ouabain additions are characteristic of an exothermic binding process. Although the individual additions by the burette system of the calorimeter last only 17.6 seconds, an unusually long reaction time of 13 min had to be chosen to reach again thermal equilibrium before the next titrant addition could be initiated. Under the chosen experimental conditions the time dependence of the observed heat response is controlled by the extremely low binding rate of the investigated inhibitor to its receptor site, which is consistent with other studies [12–14], and not by the response time of the calorimeter. A quantitative evaluation of this titration by employing the MicroCal data analysis system, based on a model consisting of a single, uniform binding site, is shown in Fig. 5B. Besides an equilibrium

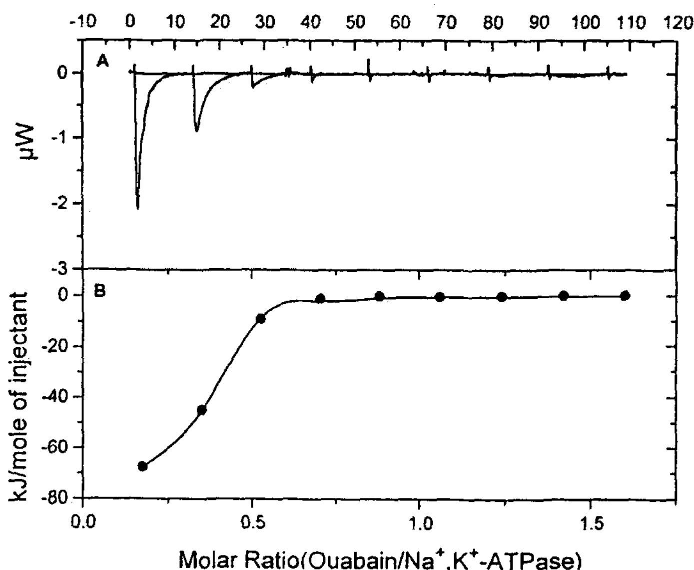

Fig. 5 A) Isothermal calorimetric titration at 25°C of 0.015 mM Na^+, K^+-ATPase in 10 mM imidazole/HCl containing 3 mM $MgCl_2$ and 3 mM phosphate *pH* 7.5 with 9 subsequent additions of 7 μl 0.5 mM ouabain solution in the same buffer medium
B) Plot of heat change *vs.* the molar ratio between ouabain and Na^+, K^+-ATPase. The solid line corresponds to a fit with $K = 7.5\times10^6$ M^{-1}, $\Delta H = -71$ kJ mol^{-1} and a stoichiometric coefficient of 0.31. Further details are given in the text

constant around 10^7 M^{-1} and a ΔH value of –75 kJ mol^{-1} as the mean value of two independent determinations, a stoichiometric coefficient of about 0.3 mol of ouabain bound per mol of enzyme ($\alpha\beta$) results. Because only a few titrant additions could be chosen prior to saturation, the accuracy of the determined equilibrium constant is limited. Higher affinities have been reported in the literature [e.g. 12, 13] by determining separately the formation and dissociation rate constants of $[H^3]$ ouabain binding and calculating the corresponding quotient to obtain the binding constant. The observed ΔH value suggests that the ouabain binding process is enthalpy driven. The formation of at least three to four intermolecular hydrogen bonds between the ligand and its receptor site upon binding to the enzyme would be consistent with the observed enthalpy value. The low value of the stoichiometric coefficient of 0.3 is considered to represent a surprising result. Although this value depends on the accuracy of the experimental protein determination, which is not very high in the case of membrane proteins, our results clearly indicate that the stoichiometric ratio is significantly below 1. Assuming at least a value of 0.5 (one ouabain per two $\alpha\beta$ enzyme molecules) as a realistic estimation, our result would be consistent with a dimer model ($\alpha_2\beta_2$), as schematically shown in Fig. 6, where one ouabain molecule is bound to only one monomer of the dimer unit. The affinity of this ligand to the second monomer unit would have to be assumed to be neglect-

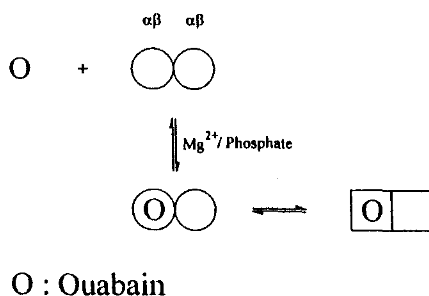

Fig. 6 Schematic illustration of the mechanism of the binding of ouabain to the receptor site of Na^+,K^+-ATPase based on a suggested dimer model

able which would be indicative of a strong negative cooperativity. Alternatively, one could claim that 50% of the enzyme molecules were inactive as a consequence of the applied isolation procedure, for example. This possibility is considered to be rather unlikely because the enzymatic activities of the samples used for these calorimetric studies belong to the highest ones that have been reported so far. Furthermore, the DSC studies did not provide evidence for a partial inactivation in the coarse of the purification procedure. However, an evaluation based not only on the Lowry protein determination, as reported above, but also on an amino acid determination seems to be desirable.

After the ouabain additions shown in Fig. 5A a sudden change in the specific heat of the solution has been observed. Further additions of ouabain resulted in the observation of an exothermic heat change which seemed not to be sensitive to the ouabain concentration. No such effect has been observed when ouabain is titrated to a phosphatidyl choline vesicle solution. Further experiments will be required to elucidate the nature of the second calorimetric effect.

Conclusions

The results clearly demonstrate that calorimetry is a suitable method to investigate the state and the thermal stability of membrane receptors under a variety of experimental conditions as well as in the presence of cofactors and ligands. This is of particular importance because membrane particles scatter light and thus give rise to turbid solutions which are not suitable for the application of many spectroscopic techniques as optical spectroscopy, for example. Even in the original membrane such as the microsomal fraction, Na^+,K^+-ATPase can be detected easily by calorimetry under conditions where an externally added enzyme substrate can only partially reach the internal catalytic site and where a characterization based on an enzymatic assay is thus only partially possible. The results provide not only information about the thermal protein unfolding but also about the effect of the membrane environment.

The DSC studies reported here demonstrate that the chosen cell filling technique can strongly influence the experimentally observed results in the case of

membrane particles, which may reflect a difficulty that is important to be considered in general when biological samples are investigated.

Titration calorimetry allows to determine the thermodynamic parameters of the interaction between ligands such as cardiac glycosides and the corresponding receptor site including information about the stoichiometry of the interaction.

Because it is very difficult to crystallize integral membrane proteins for the purpose of X-ray crystal structure analysis it appears to be important to have a suitable method such as calorimetry available that allows to characterize the uniformity of the folding and the degree of order in its local environment as well as the degree of interaction with ligands or stabilizing agents of this class of proteins before carrying out crystallization experiments.

* * *

The authors are grateful to E. Lewitzki, H. Ruf, E. Schick and F. Thommen for stimulating discussions, to A. Schacht for skilfull enzyme preparations and to the German Research Foundation for financial support (SFB 169).

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