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MEMBRANE PROTEINS IN THIN FILMS
A DSC study

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Molecular functions and structural changes of membrane proteins in an aqueous environment can be elucidated by reaction-induced FTIR difference spectroscopy upon photolysis of caged compounds. The achieved detection of IR band changes even due to single amino acid residues is, however, only possible in the presence of very high protein concentrations, implying that a low water content must be present. In general, the films are formed by controlled dehydration of membrane protein suspensions at reduced pressure and low temperature. For the retention of enzymatic activity of Na,K-ATPase, for example, a cosolvent such as glycerol is required. In order to interprete the results obtained by FTIR spectroscopy, it is important to know whether essential properties of the proteins such as hydration are changed upon film formation. Therefore, a differential scanning calorimetry (DSC) study has been carried out with purified Na,K-ATPase and Ca-ATPase in suspension, in form of pellets obtained by high-speed ultracentrifugation and in thin films. As relevant thermoanalytical properties, the endothermic denaturation transitions of the proteins have been studied.

For Na,K-ATPase in the presence of 20% glycerol as cosolvent, a single, comparatively narrow endothermic and irreversible denaturation transition with a denaturation enthalpy of about 1.7 MJ mol^{-1} and transition temperatures of about 65 and 70°C is found in concentrated suspension and in the state of the pellet, respectively. In the case of thin films suitable for IR spectroscopy, a characteristic change is observed in a reproducible manner. The enthalpy change of the remaining transition around 70°C is reduced but an additional transition at about 77°C is observed. Based on control experiments, the new high temperature transition is attributed to a partially dehydrated state of the protein. Furthermore, a comparatively broad endothermic transition around 20°C is found under conditions of high protein concentrations (film), which is tentatively assigned to a transition of the lipid environment of this integral membrane protein. Similar results are found for Ca-ATPase films. In the absence of glycerol, the deoxycholate treated enzyme in suspension exhibits a narrow endothermic main transition at 52°C with a denaturation enthalpy around 0.9 MJ mol^{-1}. For the film of this protein, two almost equally large endothermic transitions are found at 59 and 77°C. Also here, the data are characteristic of partial protein dehydration. These results show clearly that DSC can easily be applied in a sensitive manner to control and characterize the integrity and hydration properties of concentrated protein samples in thin films.

Keywords: *Ca-ATPase, DSC, IR spectroscopy, membrane proteins, Na,K-ATPase, protein film, protein hydration*

Introduction

Na,K-ATPase, the receptor of cardiac glycosides, is an integral membrane enzyme in plasma membranes. Such proteins penetrate the membrane and are surrounded by lipid molecules, as shown schematically in Fig. 1. In contrast to globular proteins, integral membrane proteins are therefore not water soluble. For their isolation and purification, it is usually required to apply surface active compounds such as detergent molecules. Therefore, many investigations have to be carried out in suspension. The biological main function of Na,K-ATPase, composed of three subunits, consists in the maintenance of the Na^+ and K^+ concentration gradients across cell membranes by performing active alkali ion transport in order to maintain the required potential. This is possible due to the fact that the free energy of hydrolysis of one ATP molecule can be converted in up-hill

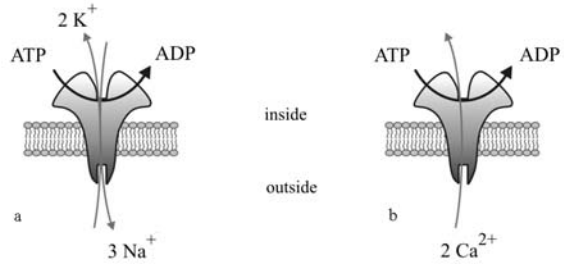

Fig. 1 Schematic illustration of the function of membrane-bound a – Na,K-ATPase and b – Ca-ATPase

or active transport of $3Na^+$ out of and $2K^+$ into the cell (Fig. 1a). The kidney enzyme, investigated here, has been isolated in form of nanoparticulate, disc-shaped membrane fragments (average size around 300 nm), containing no other protein. Ca-ATPase is an integral membrane enzyme in sarcoplasmic reticulum of muscle and consists of a single subunit. It provides

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the active transport of $2Ca^{2+}$ per hydrolyzed ATP molecule (Fig. 1b). This protein is isolated in form of closed vesicles with an average diameter of about 100 nm. In contrast to Na,K-ATPase, high resolution crystal structures have so far been reported only for Ca-ATPase [1], as shown in Fig. 2a. The membrane embedded region of the protein consists of ten α-helical transmembrane segments (*M*), which contain mainly hydrophobic amino acid residues. Some of the polar side chains in the central part of this transmembrane region form the binding sites for two Ca^{2+}. More hydrophilic parts are exposed to the aqueous solution on both membrane sides, the larger part (*N*) consisting of the ATP binding site. Only a comparatively small number of water molecules (red dots) can be localized in the transmembrane part of the protein, which is also shown in Fig. 2a. Na,K-ATPase is thought to exhibit similar structural features.

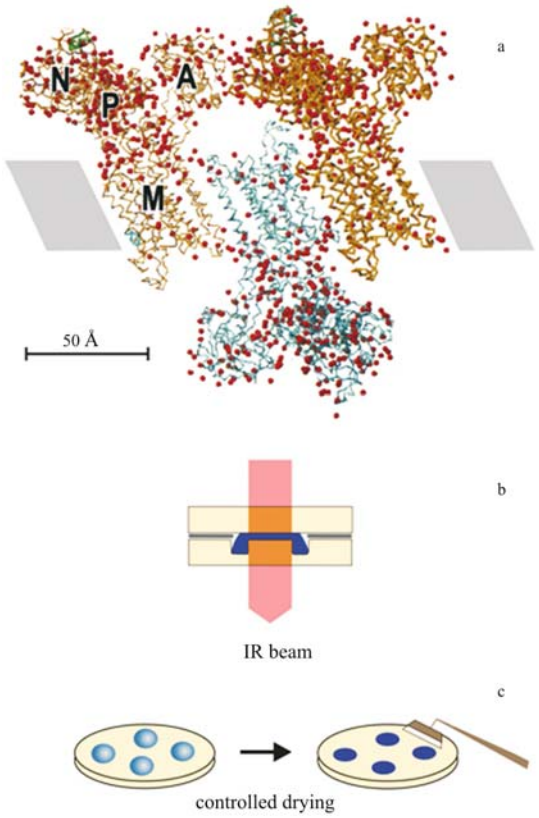

Fig. 2 a – Protein molecules in the crystal of Ca-ATPase, modified according to a figure in [1]. Grey pattern mark the assumed area of the lipid membrane. Localized water molecules are indicated by red dots. M represents the transmembrane region with the central binding sites for Ca^{2+}. Nucleotides such as ATP are bound in region N; b – Schematic illustration of an IR cell system consisting of two CaF_2 windows (yellow) and a spacer (grey) for reaction induced difference spectroscopy. The film is shown in blue colour; c – formation of protein films for calorimetric studies by concentrating droplets of membrane protein suspensions on a CaF_2 window and subsequent collection

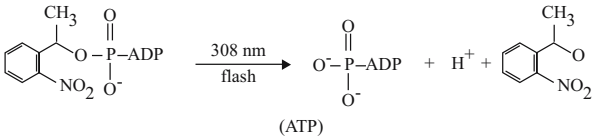

Fig. 3 Liberation of ATP by photolytic cleavage from caged ATP, a masked, inactive precursor

Enzymatic functions of proteins in thin films can be investigated by reaction-induced FTIR difference spectroscopy [2–4]. An enzymatic process is induced by the liberation of an active compound upon an UV flash from an inactive, protected derivative. A typical example is shown in Fig. 3, related to the release of ATP from NPE-caged ATP, which takes place in the ms time range. Changes of protein secondary structure and protein microenvironment can subsequently be investigated in a direct manner by FTIR difference spectroscopy. The detection of changes of IR bands, even as sensitive as due to single amino acid residues, is however only possible in the presence of high protein concentrations, implying that the water content is comparatively low. For this purpose, techniques have been developed where thin protein films with thicknesses around 5 μm and protein concentrations up to about 1 mM can be investigated by IR difference spectroscopy in a CaF_2 cell arrangement [2–4], as shown in Fig. 2b.

Such films can be formed by controlled dehydration of concentrated membrane protein suspensions at low temperature and reduced pressure. For the retention of enzymatic activity of Na,K-ATPase, for example, a cosolvent such as glycerol is required [3, 4]. Ca-ATPase, on the other hand, can also be concentrated in the absence of cosolvents and remains enzymatically active [2]. For the interpretation of the results obtained by IR difference spectroscopy from such films, it is important to know whether essential properties of the proteins such as hydration are changed markedly upon concentration. Changes in hydration may be accompanied by structural changes, leading to artificial reference states. This can lead to IR difference spectra that are not characteristic of the protein's unperturbed native structure. Thus, it is important to investigate the general properties of a protein in its concentrated film state by employing a suitable method.

Since concentrated protein films as used in reaction-induced IR difference spectroscopy [2] cannot be investigated directly with optical methods such as CD spectroscopy, for example, thermoanalytical

studies are carried out here by employing differential scanning calorimetry (DSC). Thus, thermal denaturation is applied as a characteristc criterion to characterize the state of proteins. It is generally known, that partial dehydration of proteins, for example due to the addition of organic solvents, leads to an increase of the denaturation temperature. Therefore, the thermoanalytical properties of Na,K-ATPase and Ca-ATPase have been investigated in suspension (before film formation) as well as in films. On the basis of this method, it is also possible to determine quantitatively the free water content of the protein film by freezing the sample for the determination of the melting enthalpy of ice in the sample.

Experimental

Chemicals

Chemicals were of analytical grade, supplied by Fluka, Merck and Calbiochem. In order to prepare solutions nominally free of K^+, the contact with the combined pH electrode for the pH adjustment has been omitted.

Enzymes

Purified membrane-bound Na,K-ATPase has been isolated from pig kidney according to [5, 6]. Details and analytical characterization such as protein determination employing the standard Lowry method are given in [6]. Enzymatic activity of the preparations under standard conditions at 37°C was 25–30 μmol P_i/min x mg protein. Deoxycholate (DOC) treated Ca-ATPase of sarcoplasmic reticulum has been obtained from rabbit skeletal muscle according to [7–9]. Further details are described in [10]. The DOC treatment leads to the removal of contaminating proteins of the basic preparation [9]. Enzymatic activity of the preparations under standard conditions at 37°C ranged between 13 and 16 μmol P_i/min x mg protein. For pellet formation, centrifugation has been done for 70 min at up to 170000 g with a Beckmann TL-100 ultracentrifuge at 4°C.

Differential scanning calorimetry (DSC) and protein film formation

For the study of both enzymes in suspension, a MicroCal MCS DSC instrument (MC), equipped with an inert cell of a volume of about 1.3 mL has been used. Evaluations have been carried out with the manufacturer's program.

For the thermal investigation of protein films, up to 30 droplets of concentrated protein suspension (30 μL for each droplet) on a CaF_2 window have been evacuated at about 7°C (Fig. 2c) under gravimetric control. After reaching the desired degree of concentration, the films are collected with the rubber blade of a cell scraper and transferred to the 30 μL aluminium sample pan. Amounts between 10 and 20 mg of protein film in the hermetically closed pans have been analyzed employing the Perkin Elmer 7 Thermal Analysis System (PE). The droplets are concentrated on CaF_2 because the corresponding IR studies are also done by using CaF_2 cells [2–4]. A heating rate correction has been performed. Evaluation of the DSC experiments have been performed with the program of the manufacturer.

Pellets of Na,K-ATPase, obtained by ultracentrifugation, have also been analyzed in 30 μL aluminium sample pans employing the Perkin Elmer 7 DSC instrumentation.

Results and discussion

Before film formation – Suspension and pellet

For suspended Na,K-ATPase in the buffer medium 100 mM HEPES/TRIS, 130 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA pH 7.27 containing 20% glycerol (high salt conditions), which corresponds to that of the corresponding film for IR-spectroscopy [4], a single and very narrow denaturation transition is found with a transition temperature of 64.5°C (Fig. 4 top). The endothermic transition is irreversible and characterized by an enthalpy change of about 1.7 MJ mol^{-1} protein. The very narrow transition is similar to the result reported in [11] and is indicative of a highly cooperative denaturation involving all three subunits of the enzyme. We have only observed considerably broader and more asymmetric transitions, as reported earlier [12], for enzyme preparations with enzymatic activities markedly lower than specified in the experimental part. The curve of the Na,K-ATPase pellet (Fig. 4 bottom) shows again a single main transition (Fig. 4 bottom) but exhibits a higher transition temperature (71.0°C) and a slightly wider transition range than observed in suspension. This is assumed to be due to an even lower water to protein ratio in the pellet than in suspension, leading to a lower degree of solvation in the case of the pellet. The transition enthalpy is comparable to that of the suspension sample. The broad transition range (Fig. 4 bottom) and the shoulder around 65°C may indicate a loss of cooperativity of the denaturation process of Na,K-ATPase, consisting of three subunits, in the state of the concentrated pellet.

DSC studies with standard, membrane-bound Ca-ATPase in suspension show a concentration

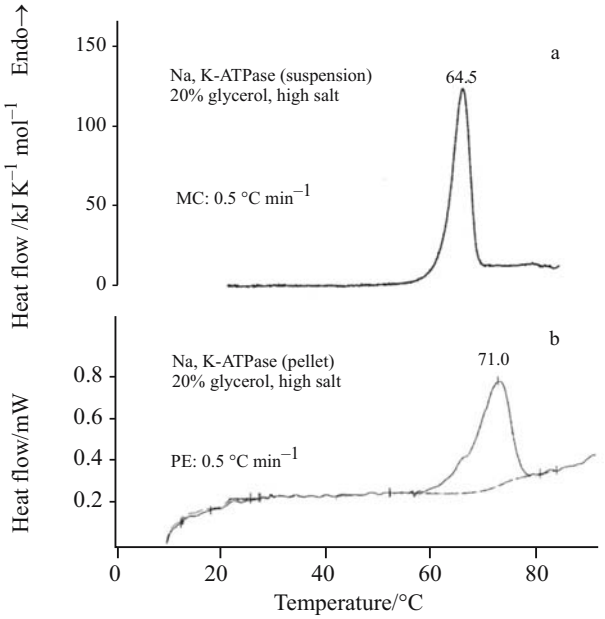

Fig. 4 DSC curves of a – Na,K-ATPase in suspension and b – in form of a pellet (bottom). Details are given in the experimental part and in the text

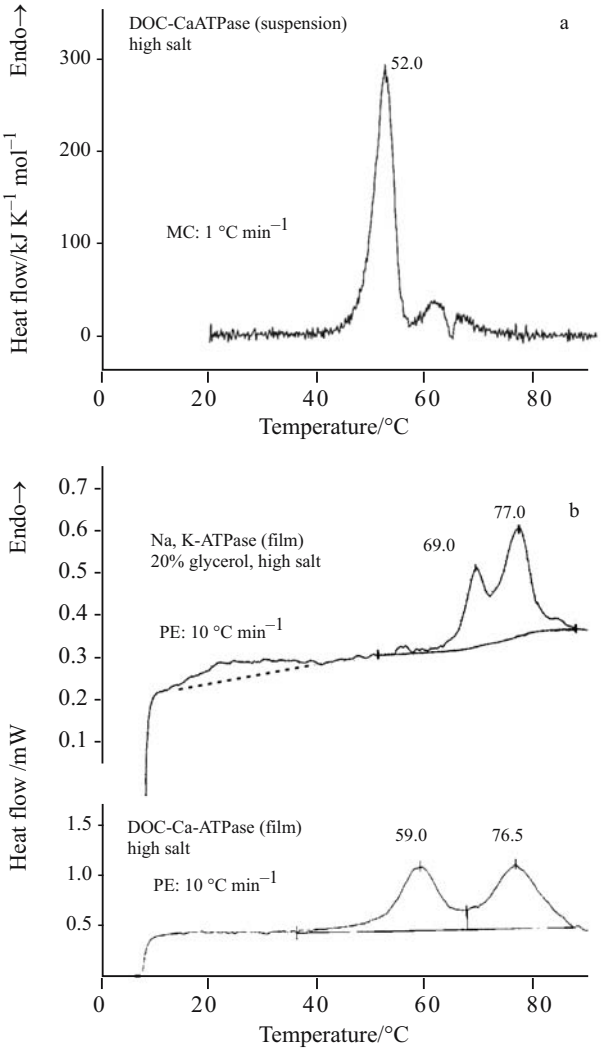

Fig. 5 a – curve of DOC-Ca-ATPase in suspension; b – curves of Na,K-ATPase and Ca-ATPase in the film. The media correspond to those of suspension and pellet. For details see experimental part, text and legend of Fig. 4

dependent, comparatively broad endothermic denaturation transition. Besides this endothermic main transition, an exothermic aggregation process has been reported [13, 14]. The corresponding superposition gives rise to a comparatively complicated DSC pattern. However, the thermogram of DOC treated Ca-ATPase in 200 mM MOPS/KOH, 210 mM KCl (total K^+ concentration 330 mM), 10 mM $CaCl_2$ pH 7.0 (high salt conditions), corresponding to the medium of IR film studies, shows a predominant single and narrow main transition at 52.0°C (Fig. 5a). The corresponding transition enthalpy is about 0.9 MJ mol^{-1}. Protein denaturation is irreversible also here. An additional, minor endothermic process with a superimposed exothermic component is observed above 58°C. No assignment of the smaller, endothermic high temperature transition around 63°C, for example whether it is due to an unknown protein contamination, is possible at present.

After film formation

After an about ten fold concentration of the Na,K-ATPase suspension in 10 mM HEPES/TRIS, 13 mM NaCl, 0.8 mM $MgCl_2$, 0.2 mM EDTA pH 7.27 containing 2% glycerol (low salt conditions) on CaF_2, the resulting medium corresponds to the high salt and glycerol conditions of the sample shown in Fig. 4. Subsequently, the films are transferred to the Al pan for the DSC experiment. The hitherto single thermal transition of Na,K-ATPase in suspension or in form of the pellet is clearly split into two separate transitions with the transition temperatures of 69.0 and 77.0°C (Fig. 5b). The lower denaturation transition temperature is close to that observed in the pellet (Fig. 4 bottom) and the total denaturation enthalpy is similar to the one determined in suspension (1.7 MJ mol^{-1}). The main denaturation process of the protein in the film occurs at an unexpectedly high temperature of 77°C. We assume that two types of protein states exist in the film, exhibiting markedly different degrees of hydration. The high temperature process is assigned to more dehydrated state or domain of Na,K-ATPase. Besides the two already described processes, an additional, smaller transition is detected around 20°C, possibly related to the lipid matrix of the membrane. The very small endotherm around 55°C is within the noise level; the minor

endothermic transition around 85°C is observed in a reproducible manner but no assignment is possible at present.

After concentrating DOC-Ca-ATPase in 20 mM MOPS/KOH, 21 mM KCl (total K^+ concentration 33 mM), 1 mM $CaCl_2$ pH 7.0 (low salt conditions) again about ten fold, the DSC experiment of the film in a medium corresponding to the high salt conditions of the suspension sample (Fig. 5a) provides a result similar to that of Na,K-ATPase. Also here, two clearly separated endothermic denaturation transitions are found (Fig. 5b). The transitions are of almost equal enthalpy change with transition temperatures of 59.0 and 76.5°C. Also here, the total denaturation enthalpy is similar to that in suspension. As for Na,K-ATPase, we assume that two different types of partially desolvated states or domains of the protein exist in the film, leading to denaturation processes occurring in different temperature ranges.

Conclusions

Both investigated membrane proteins show clearly two distinct denaturation transitions in the state of the film. This is attributed either to a loss of cooperativity during the denaturation process or to the existence of different degrees of hydration of the proteins. The observed transition temperatures of Na,K-ATPase and of Ca-ATPase are unusually high and thus indicative of marked partial desolvation. The splitting of the single transition, as observed in suspension and in the pellet of Na,K-ATPase, could be due to two type of states or domains exhibiting different degrees of desolvation, where the higher temperature transition corresponds to the lower degree of solvation.

The fractions of the proteins exposed on both sides of the membrane, which are preferentially located in the aqueous environment, are associated with a considerably larger number of water molecules than the transmembrane part. This follows from the crystal structure determination [1] and is illustrated in Fig. 1a (cf. red dots). It is therefore likely that these exposed domains exhibit a larger degree of desolvation in the state of the film than the domain embedded in the nonpolar lipid membrane part. Therefore, partially desolvated outer domains may preferentially contribute to the high temperature transition. Similar partial dehydration phenomenons are likely to exist also in protein crystals.

Partial dehydration of domains can easily be accompanied by conformational changes of the protein, concerning also its secondary structure. This can markedly affect the results of FTIR difference spectroscopy. For the purpose of safe IR interpretations, it is therefore important to check the hydration properties of the investigated proteins in such films. Under the conditions described here, the functions of Na,K-ATPase and Ca-ATPase are preserved.

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