# Secondary Structure of Heart Sarcolemmal Proteins During Interaction with Metallic Cofactors of $(Na^+ + K^+)$-ATPase

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**Abstract.** The secondary structure of membrane proteins was studied in rat heart sarcolemma by circular dichroism under conditions of interaction with metallic cofactors of $(Na^+ + K^+)$-ATPase at their optimal concentrations and under metal free conditions. Approximately 80 per cent of polypeptide chains in the membrane were organized in $\alpha$-helical structure. Upon stabilizing the $E_1$. Na conformation state of $(Na^+ + K^+)$-ATPase by $Mg^{2+}$ and $Na^+$ ions, only a slight increase in the protein $\alpha$-helix content (to 83 per cent) was observed. On the other hand, simultaneous addition of $Mg^{2+}$ and $K^+$ ions resulting in the establishment of the $E_2$ . K conformational state of the enzyme, was followed by a significant decrease in the membrane protein helicity (to 72 per cent). The presence of all three metallic cofactors of $(Na^+ + K^+)$-ATPase did not induce any further conformational change in sarcolemmal proteins as compared to the state induced by the interaction with $Mg^{2+}$ and $Na^+$ ions. In contrast to results obtained with $Mg^{2+}$ ions, the interaction of $Na^+$ with the sarcolemmal membranes led to a considerable decrease and that of $K^+$ to a significant increase in $\alpha$-helicity of the membrane polypeptides. These findings have confirmed the regulatory role of magnesium in transition of the conformational state from $E_1$ to $E_2$ in the reaction sequence of $(Na^+ + K^+)$-ATPase. Specific modulation by $Na^+$ and $K^+$ of the helicity of sarcolemmal proteins in the presence of $Mg^{2+}$ and in the absence of ATP might be considered as a preprint of conformational changes which will occur in the presence of ATP.

**Key words:** Protein conformation — Heart sarcolemma — Circular dichroism — $(Na^+ + K^+)$-ATPase

## Introduction

$(Na^+ + K^+)$-ATPase is responsible for the transport of $Na^+$ and $K^+$ ions across the plasma membrane against concentration gradients, using the energy derived from ATP hydrolysis (Skou 1965).

This enzyme has two subunits and it probably acts in its $\alpha_2\beta_2$ dimer form. The role of the smaller, $\beta$-subunit has not yet been understood. The larger, $\alpha$-subunit contains the site of ATP hydrolysis, and an aspartate residue on this subunit is phosphorylated by the $\gamma$-phosphate of ATP as an intermediate in the turnover cycle (Jørgensen 1982). In the turnover cycle, two principal cation-induced conformations of the $\alpha$-subunit, the sodium bound form $E_1$ . Na and the potassium bound form $E_2$ . K, have been defined by two different patterns of tryptic cleavage, by inactivation of $(Na^+ + K^+)$-ATPase (Jørgensen 1975, 1977), as well as by intrinsic tryptophan fluorescence (Karlish and Yates (1978).

$(Na^+ + K^+)$-ATPase is strongly associated with membrane lipids. In a highly purified preparation from the outer medulla of rabbit kidney, 382 mol of phospholipids, 67 mol of free fatty acids, 9, 16 and 12 moles of mono- di- and tri-acylglycerols, as well as 249 and 19 moles of free and esterified cholesterol bound per mol of $(Na^+ + K^+)$-ATPase were detected (Peters et al. 1981a). However, results obtained in investigation of lipids bound to the molecule of $(Na^+ + K^+)$-ATPase are limited by the purification techniques employed. Ionic detergents bind to hydrophylic portions of the membrane protein, whereas non-ionic detegrents break lipid-lipid and lipid-protein interactions (Jørgensen 1982). Thus, detergens may influence the conformation of the solubilized enzyme protein.

In the present work, the secondary structure of $(Na^+ + K^+)$-ATPase in rat heart sarcolemmal vesicles was investigated without any enzyme solubilization.

## Materials and Methods

Male Wistar albino rats (180—220 g) were used in all experiments. Membrane fraction enriched in sarcolemma was isolated essentially by the method of hypotonic shock according to McNamara et al. (1974) with a small modification in treatment with 0.6 mol . $l^{-1}$ NaI for 10 min, that was used instead of LiBr treatment, and the subsequent KCl treatment was omitted. The $1000 \times g$ sediment after NaI treatment was washed once in buffer containing 10 mmol . $l^{-1}$ TRIS—HCL and 1 mol . $l^{-1}$ EDTA pH = 7.4, and twice in the same buffer without EDTA. The latter buffer was also used to resuspend the final membrane fraction. Our modification of the technique was similar to that described by Kostka et al. (1981).

Protein concentration was determined by the method of Lowry et al. (1951). $(Na^+ + K^+)$-ATPase activity was estimated by incubating 100 μg of membrane proteins in 1 ml of medium containing 50 mmol . $l^{-1}$ TRIS pH = 7.4; 5 mmol . $l^{-1}$ $MgCl_2$; 100 mmol . $l^{-1}$ NaCl; 10 mmol . $l^{-1}$ KCl in the presence or absence of 1 mmol . $l^{-1}$ ouabain. Reaction was started by the addition of ATP (final concentration 4 mmol . $l^{-1}$). $Mg^{2+}$-ATPase activity was determined in the presence of 5 mmol . $l^{-1}$ $MgCl_2$ in the same way. Enzyme reaction was started at 37 °C, following 5 min preincubation and 10 min incubation, and it was stopped by the addition of 1 ml of 12 per cent ice-cold trichloroacetic acid. The values of $P_i$ were estimated by the method of Taussky and Shorr (1953). $(Na^+ + K^+)$-ATPase activity was expressed as the difference between $P_i$ splitted in the presence of $Na^+$, $K^+$ and $Mg^{2+}$, and in the presence of $Mg^{2+}$ ions alone.

The orientation of sarcolemmal vesicles was determined by the method of Bers et al. (1980).

Vesicles (approx. 1 mg protein/ml) were treated with SDS (0.3 mg/ml) for 20 min at 20 °C and diluted 10-fold with the enzyme-assay medium. The orientation of membrane vesicles was established from the ratio of ($Na^+ + K^+$)-ATPase activities in SDS-treated and untreated membranes, respectively. Ouabain-sensitive ($Na^+ + K^+$)-ATPase was used as a marker to assess the relative purity of the sarcolemmal fraction.

The protein conformation in sarcolemmal membranes was studied by circular dichroism (CD). CD spectra were measured with a Jasco 40 C dichrograph under constant nitrogen flush. The instrument was calibrated with d-10-camphorsulphonic acid. Measurements were performed with membrane samples containing 0.2 mg protein per ml at 30 °C in a cell with a path length of 1 mm. Data recorded at 210 to 240 nm were expressed in terms of the mean residue ellipticity (in mdeg . $cm^2$ . $dmol^{-1}$). Mean weights of amino acid residues were all set to an average value of 110 daltons. CD measurements of the membrane vesicles and absorption spectra of the solubilized membranes were all measured under identical ionic conditions using the same instrument. The sarcolemmal membranes were solubilized in a buffer-trifluoroethanol (1 : 4) mixture. Final concentrations of proteins and cations in solubilized membranes were the same as in the CD measurements. Corrected CD spectra for membrane suspensions were calculated from the original membrane CD spectra and the absorption spectra of the solubilized state, using a computer-assisted method developed by Soós and Fajszi 1984, as follows:

The content of the helical form of soluble proteins was established using following equation (Siegel et al. 1980):

$$f_H = \frac{\Theta_M - C}{\Theta_H} \tag{1}$$

where: $f_H$ is the fraction of the $\alpha$-helix; $\Theta_H$ is the mean residue ellipticity of a pure helical system (protein of polypeptide) at a given wavelength; $\Theta_M$ is the measured mean residue ellipticity at a given wavelength; and $C$ is the spectral contribution of beta and random forms.

To calculate alfa-helix contents of proteins in membrane vesicles Eq. (1) was modified by a wavelength-dependent flattening correction factor:

$$\Theta_M = Q_B(f_H\Theta_H + /1 - f_H/C) \tag{2}$$

where: $Q_B$ is the wavelength-dependent flattening correction factor different for different membrane models. For vesicles, $Q_B$ is obtained from:

$$Q_B \text{ vesicle} = e^{-(\alpha/2)} - \frac{\alpha}{2}\int_{\alpha/2}^{\infty} \frac{e^{-y}}{y}\,dy \tag{3}$$

where: $\alpha = A_{sol}/QM$ is the absorbance at the average particle thickness; $A_{sol}$ is the optimal density in the solubilized state; $Q = v/V$

where: $v$ is the total shell volume; $V$ is the total volume of the suspension; $M$ is the path length/average particle thickness; and $QM$ is the average number of particles in the light path.

## Results

The activity of ($Na^+ + K^+$)-ATPase in homogenate and in the final sarcolemmal fraction was $0.39 \pm 0.10$ and $11.2 \pm 0.76$ µmol $P_i$ . $mg^{-1}$ protein . $h^{-1}$, respectively. This means a 39-fold enrichment of the final membrane fraction in the sarcolemma. The orientation of membrane vesicles in the final fraction was $79 \pm 9$ per cent right side out as revealed from experiments with SDS treatment described by Bers et al. (1980).

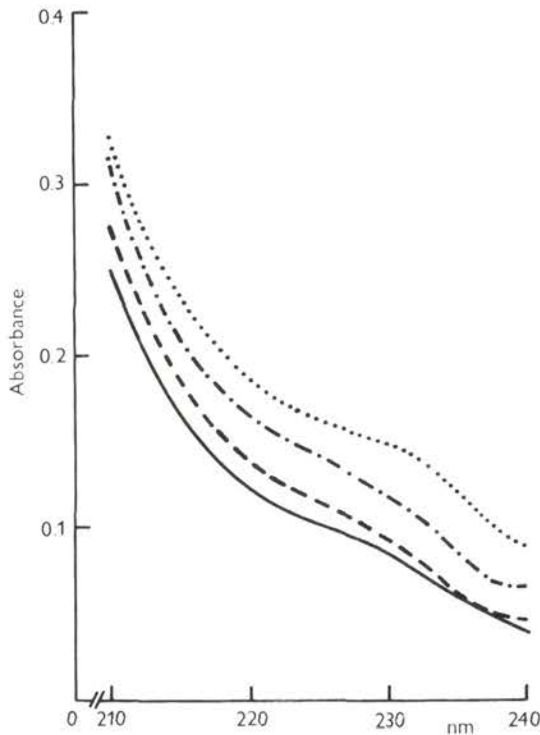


**Fig. 1.** Absorption spectra of solubilized heart sarcolemmal membranes in the presence or absence of $Na^+$, $K^+$ and $Mg^{2+}$ ions. For each measurement 200 μg of membrane protein was solubilized with concentrated 2,2,2-trifluoroethanol. The solubilized sample was adjusted with TRIS-HCl buffer to a final concentration of the latter of 50 mmol . $l^{-1}$ and a final volume of 1 ml. Concentrations of cations: $Na^+$ – 100 mmol . $l^{-1}$; $K^+$ — 10 mmol . $l^{-1}$; $Mg^{2+}$ — 5 mmol . $l^{-1}$. For other details concerning the technique of measurement, see Materials and Methods. The presented curves are representative tracings from typical measurements; dotted: Solubilized sarcolemmal membranes SL + Na; broken: $SL + Na^+ + K^+ + Mg^{2+}$; dashed: $SL + K^+$ or $SL + Mg^{2+}$; full: metal-free SL.

In addition to routine electronmicroscopic investigations, the contamination of the sarcolemmal fraction by other subcellular systems was also checked by determining following marker enzyme activities: Specific activity of $Mg^{2+}$-ATPase established in the presence of 5 mmol . $l^{-1}$ $MgCl_2$ was $22.10 \pm 0.61$ μmol $P_i$ . $mg^{-1}$ protein . $h^{-1}$ with a sensitivity to oligomycin (5 μg/ml) below one per cent. The (Mg + Ca)-ATPase activity established in the presence of 2 mmol . $l^{-1}$ $MgCl_2$ and 0.1 mmol . $l^{-1}$ $CaCl_2$ was only $0.35 \pm 0.20$ μmol . $P_i$ $mg^{-1}$ protein . $h^{-1}$. From the point of view of ATPases, this practically ruled out any significant contamination of the sarcolemmal fraction by mitochondrial membranes, sarcoplasmic reticulum or myofibrils.

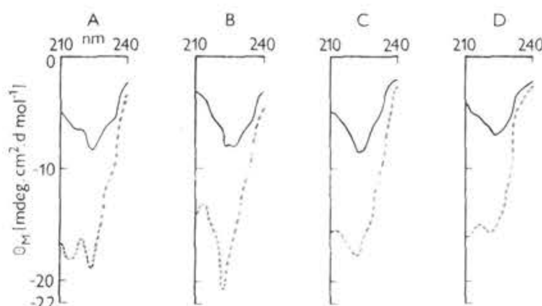


**Fig. 2.** Sarcolemmal protein circular dichroism spectra in the presence and absence of single metallic cofactors of $(Na^+ + K^+)$-ATPase. Solid lines: spectra obtained with non-solubilized, integrated membrane vesicles. Dashed lines: spectra corrected for protein contents. For concentrations of cations, membrane fraction and the applied buffer system, see legend to Fig. 1. For details concerning the technique of measurement and the method of correction, see Materials and Methods. The presented curves are representative tracings from typical measurements. *A*: Metal-free SL; *B*: SL + $Na^+$; *C*: SL + $K^+$; *D*: SL + $Mg^{2+}$.

Sarcolemmal membranes solubilized in trifluoroethanol and preincubated in the absence (metal-free state) and presence of stimulatory cations exhibited considerable differences in absorption spectra. Only results obtained in the presence of 10 mmol . $l^{-1}$ KCl and/or 5 mmol . $l^{-1}$ $MgCl_2$ yielded identical absorption curves (Fig. 1).

Figures 2 and 3 illustrate CD spectra obtained from the sarcolemmal fraction incubated under different ionic conditions. ATP was not present in any of the experiments. Corrected spectra (dotted lines) were obtained using Eq. (2). To select an appropriate $Q_B$ factor, a computer program based on Eq. (3) was applied pointing out the most suitable $Q_B$, value, from $10^4$ possibilities, i.e. a $Q_B$ value giving the smallest S.E.M. for the corrected spectra. The alpha-helix contents in membrane proteins for all experimental situations (Figs. 2 and 3) were established using Eq. (2) in a form adapted for accounting of $f_H$.

Results shown in Fig. 4 revealed that, when tested separately, both $Na^+$ (100 mmol . $l^{-1}$) and $Mg^{2+}$ (5 mmol . $l^{-1}$) interacted with sarcolemmal proteins by decreasing the alpha-helical contents from 80 per cent when metal ions were present. Conversely, potassium at a concentration of 10 mmol . $l^{-1}$ alone or in mixture with the above concentrations of $Na^+$ and $Mg^{2+}$ raised the alpha-helix contents to 84 and 83 per cent, respectively. Nevertheless, none of these changes have been found to be statistically significant. The highest alpha-helix content was observed when $Na^+$ and $K^+$ were present simultaneously, however it did not differ from that measured in the presence of both $Mg^{2+}$, $Na^+$ as well as $K^+$ ions. In the presence of both sodium and magnesium, the alpha-helical contents increased up to

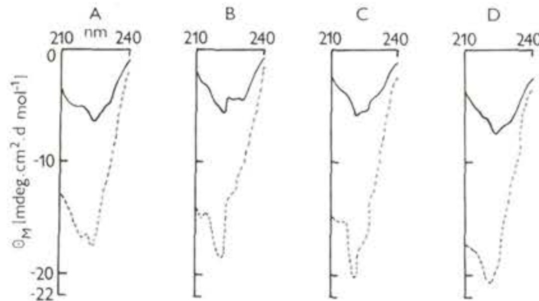


**Fig. 3.** Sarcolemmal protein circular dichroism spectra in the presence of metallic cofactors of $(Na^+ + K^+)$-ATPase in various combinations. Solid lines: spectra obtained with non-solubilized, integrated membrane vesicles; Dashed lines: spectra corrected for protein contents. For concentrations of cations, membrane fraction and the applied buffer system, see legend to Fig. 1. For details concerning the technique of measurement and method of correction, see Materials and Methods. The presented curves are representative trancings from typical measurements: *A*: $SL + Mg^{2+} + Na^+$; *B*: $SL + Mg^{2+} + K^+$; *C*: $SL + Mg^{2+} + K^+ + Na^+$; *D*: $SL + Na^+ + K^+$.

83 per cent whereas the replacement of $Na^+$ by $K^+$ in this incubation medium resulted in a decrease in alpha-helix in the sarcolemmal membranes to 72 per cent.

## Discussion

In studies of conformation changes of $(Na^+ + K^+)$-ATPase generally highly purified and/or solubilized enzyme preparations have been used (Karlish and Yates 1978; Chetverin et al. 1980; Jørgensen 1975). However, other studies revealed that detergents used in isolation procedures may influence the conformation of the enzyme under study. Foussard—Guilbert et al. (1982) reported SDS or deoxycholate to induce several irreversible alterations in the kinetic properties of $(Na^+ + K^+)$-ATPase involving changes in $K_M$, phosphorylation of the $E_1$ conformation state of the enzyme, cooperativity between the potassium binding sites, etc. On the other hand, another less invasive detergent, saponin, seems to affect the kinetic properties of the native enzyme to a lesser extent acting rather as an unmasking agent (Foussard—Guilbert et al. 1982). Nevertheless, detergent-induced changes in the secondary structure of the enzyme cannot be completely ruled out in any isolated preparation. Therefore, in the present study investigating by CD spectra the structural changes which will occur during the interaction of metallic cofactors of $(Na^+ + K^+)$-ATPase with the sarcolemmal membranes, integrated membrane vesicles were used. According to the recent knowledge, the sodium-potassium antiport secured by $(Na^+ + K^+)$-ATPase necessarily involves both ATP-induced and cation-induced conformation changes (Repke et al. 1983). Trying to disting-

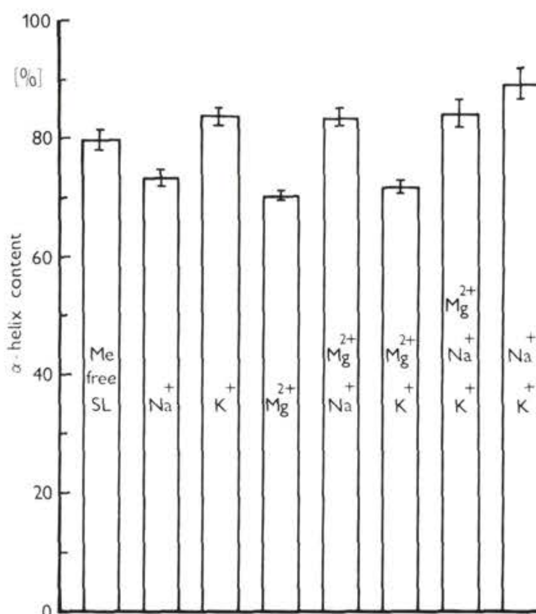


**Fig. 4.** Secondary structure of sarcolemmal proteins in the presence of different metallic cofactors of $(Na^+ + K^+)$-ATPase compared to the total $\alpha$-helical structure of poly-L-lysine. The alpha-helix content was computed from the corrected circular dichroism spectra using Eq. (2) (see Materials and Methods). For reaction conditions, see Figs. 1, 2 and 3. Results are means ±S.E.M. (6—12 measurements). All changes representing a decrease in the alpha-helix contents as compared to the metal-free state were significant at $p<0.01$.

uish between these two different types of conformation changes, our experiments were performed in the absence of ATP. Results were evaluated using a computer-assisted mathematical method for interpretation of membrane CD spectra in terms of the alpha-helical content (see Materials and Methods).

In our experiments, no considerable differences in the helicity of membrane proteins could be observed between the metal free state and that in the presence of both magnesium and sodium ions. It was supposed that, in TRIS buffer and in the presence of $Mg^{2+}$ and $Na^+$ ions, the membrane-bound $(Na^+ + K^+)$-ATPase will predominantly be in the $E_1$ . Na conformation, and in metal-free state in the $E_1$ form, whereby TRIS is bound to the $Na^+$-binding site (Rempeters and Schoner 1983). Such a finding would suggest that the transition from $E_1$ to $E_1$. Na conformation of $(Na^+ + K^+)$-ATPase does not involve any change in the membrane protein helicity.

This interpretation confirms the results obtained by Karlish and Yates (1978) who used tryptophan fluorescence technique in the presence of TRIS buffer.

According to these authors TRIS buffer itself conserves the $E_1$ conformation of $(Na^+ + K^+)$-ATPase; hence the same might also happen in the metal-free sarcolemma. The addition of $Mg^{2+}$ and $K^+$ ions to the sarcolemmal vesicles, resulting in an alteration in conformation (to $E_2 . K$) in the same TRIS containing system, led to a significant diminution (by 11 per cent) in the alpha-helix contents of sarcolemmal proteins. The contribution of the $(Na^+ + K^+)$-ATPase molecule to this diminution may represent 2—3 per cent as judged by measurements of intrinsic tryptophan fluorescence and the contents of tryptophan residues in the $(Na^+ + K^+)$-ATPase molecule (Chetverin et al. 1980). Assuming that changes in helicity concerning the $(Na^+ + K^+)$-ATPase molecule will involve only the reactive alpha-subunit of the enzyme which contains 966 amino acid residues (Peters et al. 1981b), it may be calculated that the respective 2—3 per cent decrease in helicity is related to approximately 26 amino acids. Among these 26 amino acids, only one or two may be tryptophan residues since the conformational transition of $(Na^+ + K^+)$-ATPase from $E_1$ to $E_2$ state involves only one or two tryptophan residues as reported by Chetverin et al. (1980). Thus, in absence of ATP, only a minor part of the alpha-subunit molecule might be involved in conformation changes during the transition of $(Na^+ + K^+)$-ATPase from $E_1 . Na$ to $E_2 . K$ form. If magnesium, potassium and sodium ions were added simultaneously, the conformation of sarcolemmal proteins was similar to that observed in the presence of $Mg^{2+}$ or $Na^+$ alone. This is in good agreement with observations of Matsui (1982) who showed that $Na^+$ and $K^+$ did not bind simultaneously to the same ATPase molecule. Our finding that both sodium and potassium ions have opposite effects on protein conformation in the absence and presence of $Mg^{2+}$ ions points to the regulatory role of $Mg^{2+}$ in the reaction mechanism of $(Na^+ + K^+)$-ATPase, as also suggested by Robinson and Flashner (1979). At the same time, our results seem to justify the concept that ion-induced conformation changes in the absence of ATP will also reflect changes in the $(Na^+ + K^+)$-ATPase molecule and that they might represent a preprint of changes in the secondary structure which would occur in the presence of ATP.

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