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Binding of monovalent cations induces large changes in the secondary structure of Na^+,K^+-ATPase as probed by Raman spectroscopy

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Raman spectra of active Na^+,K^+-ATPase from pig kidney in media containing Na^+ (E_1), K^+ (E_2) or without exogenous ions (E_1 conformation) were recorded in order to calculate the changes in the enzyme's secondary structure induced by binding of monovalent cations. It is demonstrated that: (i) K^+ binding to the E_1 form of the enzyme leads to conversion of ~100 peptide groups from the β-structure to α-helical conformation; (ii) the transition is reversible and fully reproducible in the $E_1 \rightarrow E_2 \rightarrow E_1$ and $E_2 \rightarrow E_1 \rightarrow E_2$ experimental schemes. Predictional calculations revealed polypeptide chain segments involved in the $\alpha \longleftrightarrow \beta$ transformations. These segments reside mainly in the two highly conserved regions of the α-subunit in the cytoplasmic domain of Na^+,K^+-ATPase. A possible role for the β-subunit is discussed.

Na^+,K^+-ATPase; Secondary structure; Raman spectroscopy; Structure prediction

1. INTRODUCTION

An asymmetrical distribution of monovalent cations between eukaryotic cells and medium is regulated by Na^+,K^+-ATPase, an enzyme which is an obligatory component in plasma membranes. Na^+ and K^+ transport via Na^+,K^+-ATPase involves protein conformational changes which have been identified for quaternary [1,2] and tertiary enzyme structures [3–6]. These experiments were carried out for many combinations of Na^+,K^+-ATPase ligands. The results were similar either to those obtained in the presence of Na^+ alone (stabilizing the so-called E_2 conformer) or to those obtained with K^+ alone (stabilizing the E_1 conformer) by either overall refolding of the molecule or relative motion of the whole domain without large perturbations in the secondary structure of the protein to explain the results.

Previous comparative studies of the E_1 and E_2 conformer secondary structures by CD and IR spectroscopy gave contradictory data [7–9]. The major difficulty with CD studies of membrane proteins is in assessing the extent of the optical artifacts introduced by the particulate nature of the protein-lipid complexes [7]. Measurements of the IR spectra in D_2O require the testing of enzyme activity in this non-biological medium, but most investigators do not perform such controls [9]. Raman spectroscopy is a very useful technique for resolving the discrepancy between CD and IR spectroscopy data. Techniques for estimating the secondary structure content of proteins in both H_2O and D_2O solutions from analysis of the laser Raman spectra are reliable and very sensitive to small changes. Moreover, estimates of protein secondary structure from Raman spectra now appear to be less susceptible to artifacts than those from CD spectra [10].

Differential analysis of the secondary structure of hydrophobic and hydrophilic domains in the α- and β-subunits of Na^+,K^+-ATPase by Raman spectroscopy was reported in [11,12]. This study deals with evaluation of the differences in secon-

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dary structure connected with the $E_1 \leftrightarrow E_2$ transitions in Na^+,K^+-ATPase by Raman spectroscopy.

2. EXPERIMENTAL

Isolation of Na^+,K^+-ATPase from pig kidney outer medulla, measurements of ATPase activity and concentrations were performed as described [13]. Enzyme with ATPase activity greater than 36 units was used. For enzyme stabilization in the Na^+ (E_1) or K^+ (E_2) forms, NaCl or KCl was added to membrane-bound Na^+,K^+-ATPase suspended in aqueous medium containing 0.3 M sucrose, 30 mM histidine, pH 7.5, until the final salt concentration was 150 mM.

To perform experiments following the schemes $E_1 \rightarrow E_2 \rightarrow E_1$ and $E_2 \rightarrow E_1 \rightarrow E_2$, NaCl or KCl was added step by step to the salt-free form of Na^+,K^+-ATPase: NaCl (up to a final concentration of 20 mM), KCl (20 mM) and NaCl (140 mM); or KCl (20 mM), NaCl (120 mM) and KCl (70 mM). Raman spectra were recorded after each salt addition.

To control enzyme stabilization in the E_1 and E_2 conformations we used the peptide maps obtained after limited trypsinolysis of Na^+,K^+-ATPase in the media described above followed by SDS electrophoresis in polyacrylamide gels [13].

The Raman instrument, details of sample preparation and recording of spectra and analysis are described in our previous papers [11,12]. We also report the scheme of the complex approach to identification of hydrophobic and transmembrane segments of Na^+,K^+-ATPase and the distribution of regular structures along the polypeptide chain.

3. RESULTS AND DISCUSSION

Raman spectra in the amide I region of the E_1 and E_2 conformers of membrane-bound Na^+,K^+-ATPase in media containing Na^+ or K^+ as well as without exogenous ions (salt-free form) were recorded (fig.1). In all media used the samples retained ATPase activity comparable to those of the original samples prior to Raman experiments. We detected large conformational differences between the E_1 and E_2 forms of the enzyme (table 1). Binding of K^+ decreases the percentage of β-structure and increases the α-helix content in comparison with the Na^+ or salt-free forms. This effect is completely reversible and reproducible as demonstrated by the $E_1 \rightarrow E_2 \rightarrow E_1$ and $E_2 \rightarrow E_1 \rightarrow E_2$ schemes (fig.1). Alteration in the enzyme's secondary structure by ~10% (table 1) corresponds to the conformational transition of more than 100 peptide groups of the protein and cannot be explained by the model that proposes minimal changes in secondary structure but motion of the entire domain of the enzyme in the process of active transport for Na^+ and K^+ across the cell membrane [14].

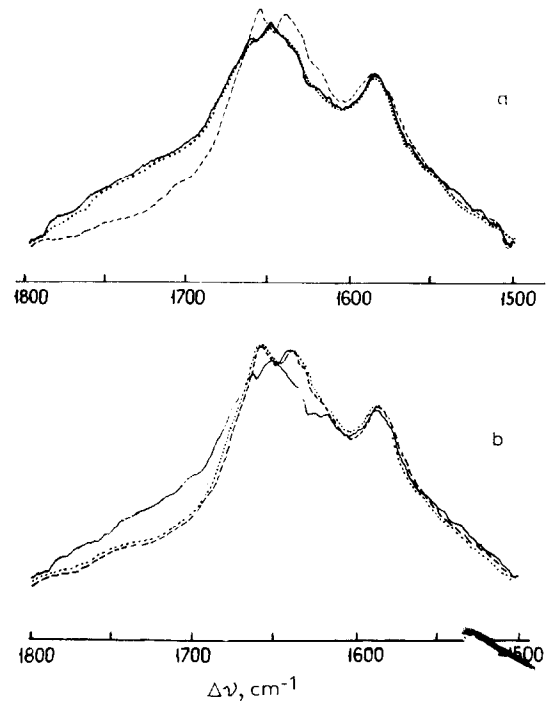

Fig.1. Comparison of Raman spectra of Na^+,K^+-ATPase conformers E_1 and E_2. (a) $E_1 \rightarrow E_2 \rightarrow E_1$ experimental scheme: (——) salt-free form (E_1), (– – –) K^+ form (E_2), (· · ·) Na^+ form (E_1). (b) $E_2 \rightarrow E_1 \rightarrow E_2$ experimental scheme: (——) Na^+ form, (· · ·) and (– – –) K^+ form.

In previous works on secondary structure calculations on the E_1 and E_2 conformers of Na^+,K^+-ATPase from the analysis of CD [7,8] and IR [9] spectra, very contradictory results were obtained, which did not agree with our data (table 1). CD spectroscopy [7] demonstrated a 7% decrease in α-helical content and an increase in percentage of antiparallel β-sheet and unordered conformation induced by K^+ binding that is the reverse of the effect found in our work. The same technique but using another method for correction of light scattering artefacts did not register significant alterations of the enzyme secondary structure induced by monovalent cations [8].

IR spectroscopic data do not reveal any large conformational changes during ion binding, but only a slight (<2%) increase in α-helical content in the K^+ form [9] (table 1). Analysis of the Raman spectra of samples deuterated as described in the

Table 1

Differences in calculated secondary structures of E_2 and E_1 conformers of Na^+,K^+-ATPase, as probed by Raman, IR and CD spectroscopy

Spectroscopy technique	Difference between E_2 and E_1 conformers (%)		
	$\Delta(\alpha$-helix)	$\Delta(\beta$-structure)	Δ(unordered conformation)
Raman (this work)	(10 ± 5)	−(8 ± 5)	−(2 ± 5)
CD [8]	−7	10	0
CD [9]	0	0	0
IR [10]	2	0	−2

Data were obtained by subtraction of the percentage of secondary structure in the E_2 conformer from that of the same structure in the E_1 conformer

IR study [9] shows no differences in the amide I regions of E_1 and E_2 [15]. An explanation for this fact can be connected with previously established inhibition of Na^+,K^+-ATPase activity and some partial reactions catalyzed by the enzyme in media containing high concentrations of D_2O, even without a deuteration procedure [16]. According to our results, ~40% inhibition of ATPase is observed when H_2O is substituted with D_2O in media. Inhibition increased to ~50% after deuteration [15]. Therefore, we have arrived at the conclusion that in the IR experiments [9] conformational alterations are negligible due to strong inhibition of ATPase activity as a result of the process of sample deuteration.

The main problem involved in the interpretation of CD spectra concerns the impossibility of correctly evaluating the changes in light scattering upon addition of Na^+ and K^+. Moreover, the techniques used for refinement of the CD spectral data are imperfect and can be sources of significant systematic error. It has been demonstrated that for the salt-free and Na^+ forms of the enzyme, the level of scattered light is low enough and the contents of the secondary structural elements quite similar to that obtained in [7]. At the same time, K^+ addition induces essential changes in intensity of the scattered light. Furthermore, in contrast to Raman measurements, we registered large spectral differences in the CD spectra upon addition of K^+ to the salt-free form of inactivated Na^+,K^+-ATPase [15].

Thus, the contradictions between Raman, IR and CD data are caused by artefacts in the latter two techniques (enzyme inactivation upon hydrogen-deuterium exchange and influence of light scattering, respectively). Estimations of secondary structural changes for the membrane-bound proteins from Raman spectra appear to be less susceptible to artefacts than those for other optical methods [11].

To understand the molecular mechanism of ATP-dependent ion pump functioning, it is very important to localize conformational changes connected with $E_1 \leftrightarrow E_2$ transitions. Unfortunately, the correct assignment of changes in the polypeptide chain seems to be impossible at present. We believe that the segments are potential candidates for participation in structure rebuilding and can be positioned by predictional techniques of data analysis of the protein amino acid sequence. This technique was employed [11,12] to calculate the distribution of regular structures along the polypeptide chain.

Application of statistical algorithms is usually accompanied by the appearance of several 'questionable' segments which can form various secondary structures. For such segments, the mean conformational parameters of different types of regular structure are very similar. Therefore, a number of additional factors (e.g. pattern of distribution of hydrophobic and charged residues, secondary structure of adjacent segments in $\beta\alpha\beta$ Rossman folds, presence of β-turn, disulfide bond formation) must be taken into account. One can propose that the questionable fragments in the protein polypeptide chain can undergo conformational transitions between various types of secondary structure with a high probability.

To identify such conformationally labile segments in Na^+,K^+-ATPase, the results of secondary structure predictions obtained from the Chou-Fasman and Garnier algorithms [11] with lower cut-off values for the mean conformational parameters of the α-helix and β-sheet were analyzed. Fig.2 shows these segments in the model of the Na^+,K^+-ATPase transmembrane organization, the proposed conformationally labile segments being located mainly in two extensive fragments of the α-subunit, 161–210 and 576–729, in the cytoplasmic domain and undoubtedly of interest for assignment of conformational changes accompanying the $E_1 \rightarrow E_2$ transition.

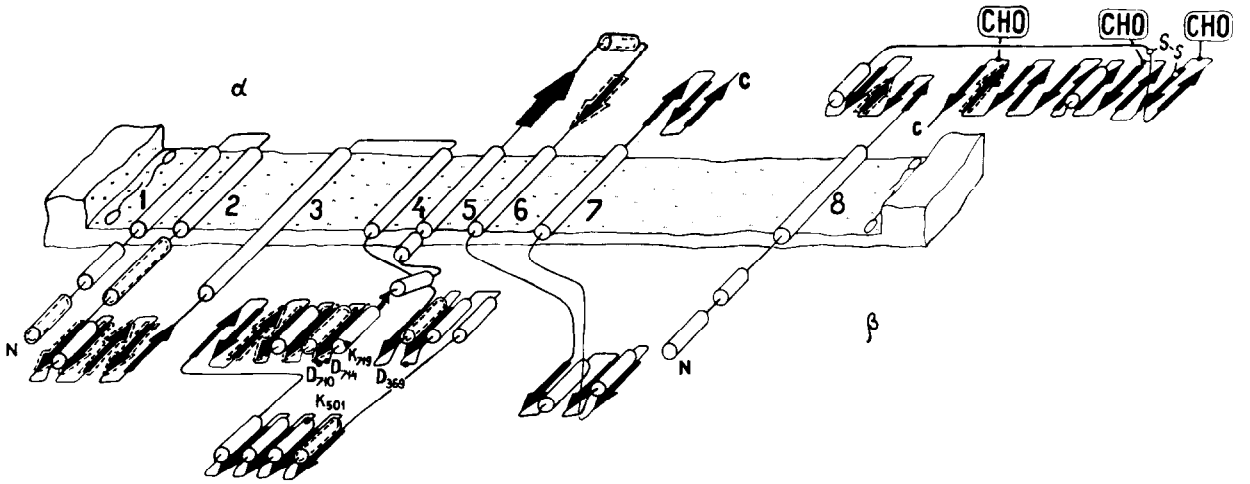

Fig.2. Na^+,K^+-ATPase secondary structure transmembrane organization based on Raman data and predictional calculations [11,12]. Cylinders and arrows correspond to α-helical segments and β-pleated sheets, respectively. Dashed lines indicate the segments that are candidates for participation in the $\alpha \leftrightarrow \beta$ transition which accompanies $E_1 \leftrightarrow E_2$ transformations of the enzyme.

It should be noted that all of these segments are included in the fragments of the protein sequence showing a high level of homology with other catalytic subunits of ATP-dependent translocases of the E_1/E_2 type [17]. One may conclude that they are involved in a general mechanism of functioning connected with the $E_1 \leftrightarrow E_2$ conformational transition.

Secondary structure evaluation studies have been performed for Ca^{2+}-ATPase from sarcoplasmic reticulum [18,19], however, with contrasting results. IR spectroscopy allowed the conclusion that the α-helical content in E_2 increases slightly compared with E_1 (consistent with the present work), but quantitative estimations were not performed [18]. At the same time, according to CD spectroscopy [19] $E_1 \leftrightarrow E_2$ transitions are not accompanied by noticeable secondary structural changes of Ca^{2+}-ATPase. If Ca^{2+}-ATPase functioning demands no significant structural changes in the enzyme secondary structure, an alternative explanation of the results obtained in the present work should be considered. It is reasonable to take into account an additional component of Na^+,K^+-ATPase, unique to this enzyme, namely the β-subunit, its role in ATP-dependent ion pumping being still unclear. One may suppose that (i) the conformational changes which accompany the $E_1 \leftrightarrow E_2$ transition affect intersubunit interactions inside the $(\alpha\beta)$ protomer or (ii) the secondary structural changes are located mainly in the β-subunit.

In order to choose between these two alternatives, experiments based on a previously developed approach to the distribution of secondary structure elements among the enzyme subunits [11,12] are now in progress.

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