

Do Sodium and Potassium Forms of Na,K-ATPase Differ in Their Secondary Structure?*

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Infrared spectroscopy in the amide I region of purified membrane-bound Na,K-ATPase preparation shows that Na⁺- and K⁺-bound forms of the enzyme have almost the same secondary structure. No difference is detected in the β -structure (pleated sheets) content. This is contrary to the statement of the recent paper (Gresalfi, T. J., and Wallace, B. A. (1984) *J. Biol. Chem.* 259, 2622-2628) where a similar preparation was examined by circular dichroism spectroscopy and it was claimed that net 7% of protein peptide groups undergo a β -sheet to α -helix conformational change upon Na,K-ATPase conversion from the K⁺ to the Na⁺ form. The discrepancy of the results is most likely caused by the particulate nature of the enzyme preparations used that could lead to optical artifacts in CD but not in IR measurements. A thorough comparison of IR spectra of these enzyme forms has revealed a very minor spectral difference which could suggest conformational perturbations, if any, of a much lower scale and another type than that claimed by Gresalfi and Wallace. The K⁺ form tends to absorb slightly more in the region of the α -helix band. This could reflect some distortion or a transition to a random coil structure of a small fraction of α -helical segments ($\leq 2\%$ protein peptide groups) upon the enzyme conversion from the K⁺ to the Na⁺ form.

Na,K-ATPase is a transmembrane enzyme which under usual conditions performs translocation of three Na⁺ ions inside and two K⁺ ions outside the animal cell with a coupled hydrolysis of one ATP molecule (1). It is natural to expect the translocation process to be accompanied by more or less significant changes in protein molecule configuration, at least near the cation-binding sites. Intensive studies during the last decade gave strong evidence of real occurrence of conformational changes in Na,K-ATPase, with the main changes found upon transition between the functionally opposite states where the enzyme displays different affinities to Na⁺ and K⁺ (2, 3). What, however, is the kind and the scale of these changes? Answering this question is of great importance for ideas on the functional mechanism of this transport system.

In the previous papers (4, 5), we have monitored the sec-

ondary structure of the membrane-bound Na,K-ATPase by infrared spectroscopy, taking advantage of the absence in the infrared region of light scattering from membrane fragments of which the enzyme preparation consisted. It has been found that the Na,K-ATPase secondary structure is the same, within experimental error, in all states acquired by the enzyme as the ATP hydrolysis cycle proceeds, including those shown to possess different conformations by other methods. From this, the number of peptide groups which could change their conformation upon transition between those states has been estimated to amount at best to no more than 3% of all the protein peptide groups (5).

However, these results seem to be unknown to the authors of a recent paper (6) who have claimed that there are extensive conformational changes upon transition between sodium and potassium forms of Na,K-ATPase. Their measurements were performed on a similar enzyme preparation but with a quite different technique of circular dichroism spectroscopy which seems to be hardly suitable in the case of particulate samples because of an intense light-scattering effect they produce in the ultraviolet region. To clear up this point, we decided to make a direct comparison of the IR spectra of these enzyme forms since this was not made in our earlier work (5).

The IR spectra of the potassium and sodium forms of Na,K-ATPase and their difference spectrum in the main amide bands' region are shown in Fig. 1. The spectra were measured after the enzyme preparation was exposed to heavy water for 24 h at 4°C. As it follows from the value of the integral intensity of amide A band (11) (Fig. 1, *inset*), there are 28% unexchanged peptide groups under these conditions. The hydrogen exchange degree remained on the same level throughout the measuring procedure. The largest peak in the spectra with the absorption maximum at 1650 cm⁻¹ corresponds to the amide I band whose shape and amplitude are determined by conformation of protein peptide groups (9, 10, 12); for the relation of other bands, see Ref. 4. Partial deuteration of protein samples, as it occurs in our case, results in shifting of the amide I components to slightly lower frequencies (9, 10, 12) without changing their integral intensities (9, 10). Thus, IR spectra of partially deuterated samples are quite suitable for observing the state of secondary structure of the whole protein molecule.

From the results presented in Fig. 1 it follows immediately that the potassium and sodium forms of Na,K-ATPase possess practically the same secondary structure, in agreement with the conclusion of our previous paper (5). At the same time, due to an improvement of conditions of measurements (higher isotopic purity of samples, better compensation by protein concentration and H₂O content, absence of any additions absorbing in the region of the amide I band, and direct recording of difference spectra of the compared samples) their reliability and reproducibility were made substantially higher than in Ref. 5; this allowed us to detect a very small spectral difference in the vicinity of the 1650 cm⁻¹ point (*bottom* of Fig. 1). Since this difference remained observable in the wide range of protein concentrations and ionic strength (see legend to Fig. 1), it seems unlikely to be an artifact.

Fig. 2A presents an IR spectrum of all regular parts of the secondary structure of the sodium form of the enzyme in the amide I band region. Data on amino acid composition and

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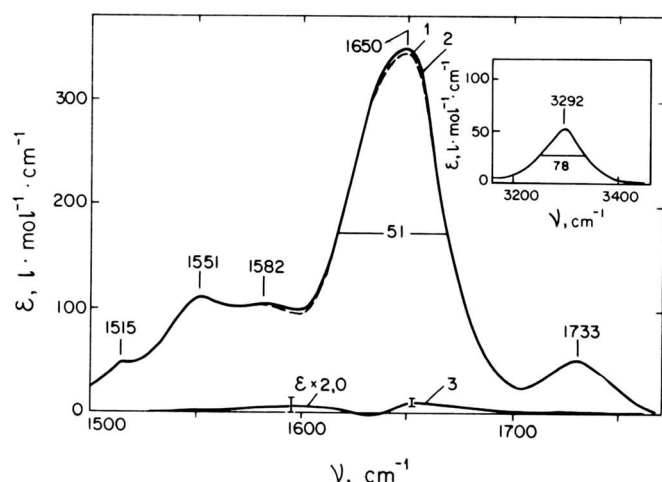


FIG. 1. Infrared spectra of Na,K-ATPase. The membrane-bound preparation of Na,K-ATPase from pig kidney isolated by the procedure (7, 8) in the presence of 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride had a specific activity of $\approx 30 \mu\text{mol}$ of P_i/mg of protein/min which was completely suppressed by ouabain and contained $\geq 95\%$ of α -plus β -subunits estimated as a fraction of the stained material after electrophoresis in the presence of sodium dodecyl sulfate and urea. It was transferred into the 25 mM Tris-DCI, pH_{app} 7.6, 1 mM EDTA (free acid) buffer by passing through a Sephadex G-25 (fine) column at 20°C . Before measurements (after 24-h storage at 4°C), the procedure was repeated. Isotopic purity of the sample obtained (by D_2O) was $\geq 99.4\%$. The initial protein concentration was $\approx 1.7\%$; the samples of smaller concentrations were made by dilution with the same buffer. To obtain the potassium and sodium forms of the enzyme, equal aliquots of the same stock suspension were mixed just before measurements with small equal aliquots ($\leq 5\%$ of the sample volume) of concentrated KCl or NaCl solutions prepared on the same buffer. IR spectra were measured with a double-beam Perkin-Elmer M180 spectrophotometer with the spectral slit width of 3 cm^{-1} in demountable fluorite cells of $120\text{-}\mu\text{m}$ path length at $20 \pm 1^\circ\text{C}$. Spectra were recorded in the absorbance mode with variable or 5-fold ordinate expansion. The exact values of protein concentrations were obtained by measuring peak intensity of the amide I band using the extinction coefficient determined in Ref. 4. Difference spectra of samples carefully compensated by H_2O absorption were measured with a 10-fold ordinate expansion in cells of exactly the same path length (the difference spectrum showed no deviation from a straight line when the cells were filled with the buffer solution). Compensation by protein concentration was better than 0.2%. When measuring original spectra, a reference cell with a path length 1% smaller than that of the sample cell was used for a better solute compensation. Other details of measuring quantitative IR spectra are given elsewhere (9, 10). 1, spectrum of the sodium form; 2, spectrum of the potassium form; 3, a 2-fold ordinate expansion of the difference spectrum obtained upon averaging of directly-in-triplicate-measured difference spectra at protein concentrations of 0.27, 0.8, or 1.6% in the presence of 10 mM or 100 mM KCl (sample cell) or NaCl (reference cell). Inset, IR spectrum of the enzyme preparation in the region of the amide A band.

amide nitrogen content of the Na,K-ATPase preparation as well as the protein concentration determined by nitrogen assay¹ were taken into account. This information allowed us to evaluate the secondary structure composition more precisely than in the earlier paper (4). The maxima of the split β -sheet band (1631 and 1688 cm^{-1}) as well as of the α -helix band (1654 cm^{-1}) are clearly seen in the spectrum of Fig. 2A, and α - and β -components can be readily isolated therefrom (see Fig. 2). This leads to the estimation (9, 10) that 22% of protein peptide groups are involved in α -helices and 22% in pleated sheets, which is close to the figures reported earlier (20 and 25%, respectively) (4).

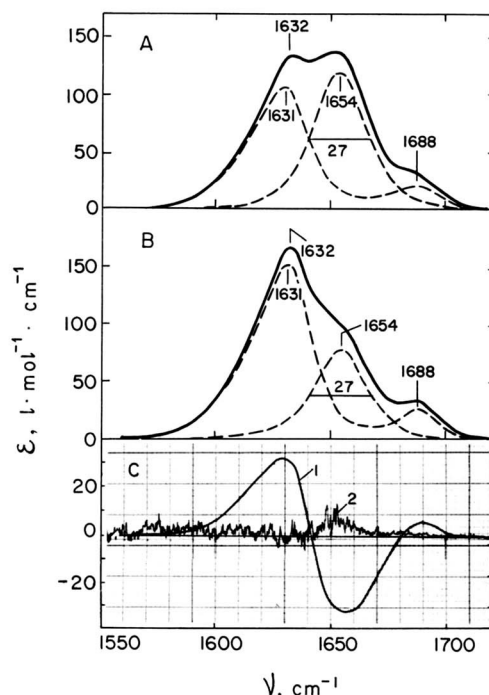


FIG. 2. Analysis of the amide I band in the IR spectrum of the sodium form of Na,K-ATPase. A, spectrum of the regular part of protein secondary structure obtained upon subtraction from spectrum 1 (Fig. 1) of absorption of amino acid residue side groups (13) and that of 55% of an unordered form (4). Bands 1631 and 1688 cm^{-1} belong to the antiparallel β -form, and that of 1654 cm^{-1} to the α -helix; B, simulated spectrum of the regular part of the potassium form obtained with the assumption that 7% of peptide groups undergo an $\alpha \rightarrow \beta$ transition upon the enzyme conversion from the sodium form (A) to the potassium form (cf. Ref. 6). C, simulated difference spectrum obtained upon subtraction of spectrum A from spectrum B (1), and experimentally measured difference spectrum (2).

Based on the above analysis, we have simulated a situation which would be observed if the conclusion of the $\alpha \rightarrow \beta$ transition involving 7% of protein peptide groups (6) was correct. An expected IR spectrum of regular structures of a "potassium form" (Fig. 2B) and a corresponding difference spectrum (Fig. 2C, curve 1) were calculated. It is obvious that this imaginary situation is far from that observed in reality. The detected difference in IR spectra of the potassium and sodium forms of Na,K-ATPase is much smaller in size than should be expected from the conclusion in Ref. 6. Moreover, in contrast to the claim that the α -helix content falls upon transition to the potassium form (6), this form tends to absorb slightly more, just in the region of α -helix absorption (Fig. 2).

If we assumed that the minor spectral difference observed (Fig. 1, curve 3, and Fig. 2C, curve 2) reflected real changes in peptide groups' conformation (that, however, could not be done conclusively because of the very small size of the effect), this would suggest a small fraction of α -helical structures, amounting to $\approx 2\%$ of protein peptide groups, to be somehow disordered (14) or converted to a random coil structure upon transition of Na,K-ATPase from the potassium to the sodium form. Such an interpretation would be consistent with our data on the intrinsic fluorescence of Na,K-ATPase (15) showing the tryptophan fluorescence spectrum of the sodium form to be slightly shifted to longer wavelengths as compared with that of the potassium form. This may be indicative of a less rigid state of protein structure in the sodium form than in the potassium form of the enzyme.

Concerning the discrepancy of our results with those of

¹ A. B. Chetverin, submitted to *Eur. J. Biochem.*

Gresalfi and Wallace (6), we cannot make a definite conclusion at present. The most likely explanation seems to be a high turbidity of membrane-bound Na,K-ATPase preparations which may lead to artifacts in measuring CD spectra in the ultraviolet region.

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