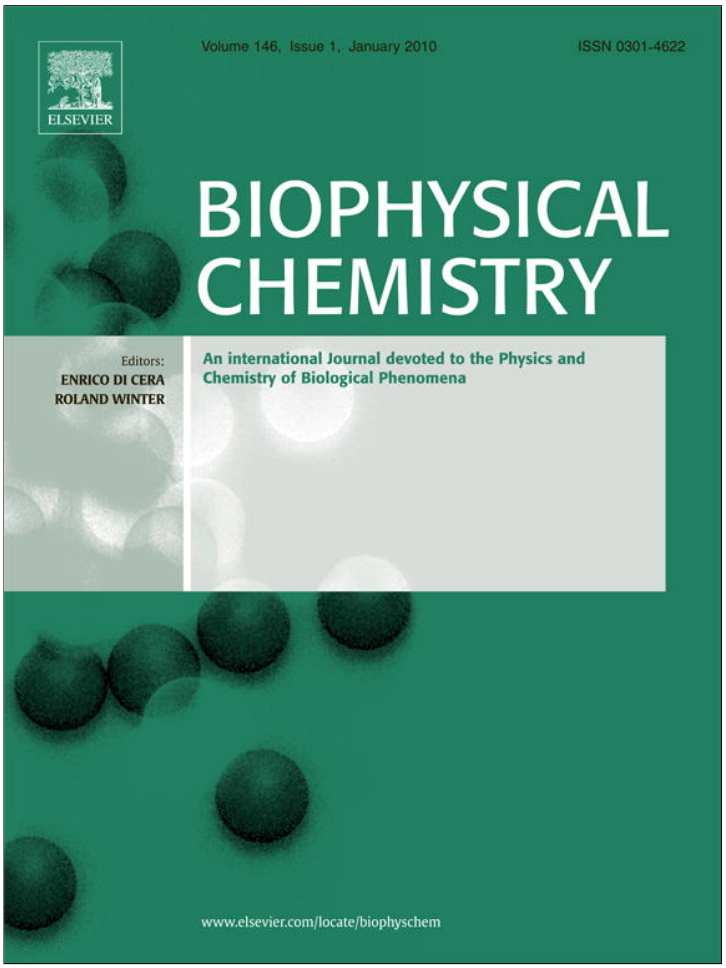

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Cytoplasmatic domain of Na,K-ATPase α-subunit is responsible for the aggregation of the enzyme in proteoliposomes

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ABSTRACT

We studied the thermal dependence of amide I′ infrared absorption and fluorescence emission of Trp residues in the Na,K-ATPase of rabbit kidney. We studied the whole enzyme solubilized with detergent, the whole enzyme reconstituted in proteoliposomes and the protein fraction that remained in the lipid membrane after the trypsin digestion of the proteoliposomes. Cooperative unfolding and aggregation with increasing temperature were observed in the whole protein, whether solubilized or reconstituted, but not in the fraction remaining after trypsinization. The protein influenced the physical state of the lipid, decreasing the temperature of the gel to liquid-crystalline phase transition and the degree of cooperativity. This study provides new information for the understanding of the processes controlling the association mechanisms that are important for enzyme function in natural membranes.

1. Introduction

Na,K-ATPase is a member of the P-type family of active cation transport proteins and is present in the plasma membrane of virtually all animal cells. The enzyme has been purified from tissues rich in the protein [1,2], and our laboratory has been studying the $(\alpha\beta)_2$ form of Na,K-ATPase from the outer medulla of rabbit kidney [3–5]. The transport function is well established, and the enzyme has an additional signal transduction function, resulting in the modulation of cell growth and adhesion [1,2,6,7].

The enzyme complex consists of two polypeptide chains. The α-subunit or catalytic chain (~110 kDa) is responsible for the enzymatic activity [5,8], and the β-subunit (~35–50 kDa) appears to play a role in the catalytic reaction and ion-pumping mechanism [8,9], in addition to a structural function. The existence of a small protein, with a single membrane segment has still to be considered. It has an N-terminal portion to the extracellular side and the C-terminal to the cytoplasm side, as do all proteins of the FXYD family. They are responsible for the fine tuning that regulates the activity of the Na,K-ATPase in the kidney through the changes in the Na^+ and K^+ affinity, although it is not necessary for the expression of either activity of the enzyme [10–12].

It has been demonstrated that the αβ form of the Na,K-ATPase is capable of both ATP hydrolysis and active ion transport [13–15]; however accumulating evidence suggests that the enzyme normally self-associates as $(\alpha\beta)_2$ dimers [3,4,16–18] or as $(\alpha\beta)_4$ tetramers [19–21].

The crystal structure [22,23] and kinetic experiments [1] support the existence of occluded Rb^+ and K^+ states. Furthermore, the crystal structure also confirmed that the α-subunits of the Na,K-ATPase and Ca-ATPase are surprisingly similar [22,23].

Significant progress in the characterization of the secondary structure and assembly of the transmembrane domain of Na,K-ATPase has been made using the FTIR technique. These studies were done with a preparation in which large parts of the extramembranous portion of the α-subunit were removed by extensive trypsinization [24].

We have previously studied the structural changes under the thermal unfolding of both the detergent solubilized $(\alpha\beta)_2$ form of Na,K-ATPase from the outer medulla of rabbit kidney and the enzyme reconstituted in DPPC:DPPE-liposomes [25–27]. These studies identified changes in the content of α-helices and β-sheet resulting either from changes in the enzyme conformation or from aggregation. However, the interpretation of experiments with solubilized Na,K-ATPase has been hampered by the relative instability of the enzyme and the aggregation occurring among α–α subunits. In this work, using FTIR and fluorescence, we have studied in detail the aggregation of the α- and β-subunits of the Na,K-ATPase reconstituted in liposome with or without trypsin treatment.

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2. Materials and methods

2.1. Material

All solutions were made using Millipore Direct-Q ultra pure apyrogenic water and all reagents were of the highest purity commercially available: Trichloric Acetic Acid (TCA); tris[hydroxymethyl] aminomethane (Tris); N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES); Adenosine 5′-Triphosphate Tris salt (ATP); bovine serum albumin (BSA), trypsin, D_2O and Dodecyloctaethyleneglycol ($C_{12}E_8$) were from Sigma. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were from Avanti Polar Lipids. Ethylenediaminetetraacetic acid (EDTA), potassium chloride, sodium chloride and magnesium chloride were from Merck. Biobeads were from BioRad.

2.2. Na,K-ATPase and proteoliposomes preparation

Solubilized Na,K-ATPase was obtained as previously described [3,5]. Proteoliposomes of DPPC:DPPE (1:1 w/w) were prepared by co-solubilization method using lipid:protein ratio (1:3 w/w) as previously described [4], resulting in a vesicular system with obtained diameters of 140 nm. The protein content was determined as described by Cornelius et al. [28].

Na,K-ATPase reconstituted in DPPC:DPPE-liposome (0.6 mg/mL) was treated with 0.2 mg/mL of trypsin during 30 min at 37 °C. The smaller polypeptide units and trypsin were eliminated by ultracentrifugation for 1 h, at 100,000 *g* and 4 °C and the pellet was re-suspended in 5 mM Tris–HCl buffer, pH 7.0 containing 150 mM KCl.

2.3. FTIR spectroscopy

Proteoliposome and trypsinized proteoliposomes were ultracentrifuged for 1 h, at 100,000 *g* and 4 °C. The pellets were washed twice with D_2O containing 0.1 M KCl.

FTIR spectra were recorded with a Nicolet Nexus spectrometer using CaF_2 windows with a 50 μm Teflon spacer in a demountable, thermostated cell. The temperature was controlled with a circulating water bath and measured with a thermocouple placed in contact with the crystal windows. The spectrometer was permanently purged with dry air to reduce the water vapor distortions. Interferograms were accumulated over the spectral range of 400–4000 cm^{-1} with a normal resolution of 2 cm^{-1}. Band narrowing by Fourier self-deconvolution (FSD) was performed with the Omnic program provided by Nicolet. The scan rate in the experiments where the temperature was increased was about 1 °C/min.

2.4. Fluorescence

Intrinsic fluorescence from the solubilized enzyme, proteoliposome and trypsinized proteoliposome (0.5 mg/mL) was measured in a Fluoromax 3P from Horiba Jobin Yvon (Edison NJ, USA) using a 3 mm optical path quartz cuvette. Tryptophan fluorescence was selectively excited with light of 295 nm wavelength. The temperature was controlled with a water circulating bath and measured directly in the solution. The results shown are the average of three experiments.

3. Results and discussion

3.1. Infrared studies

We used infrared spectroscopy to study the structure and conformational changes of the whole Na,K-ATPase reconstituted in proteoliposomes and of the protein fractions that remained bound to the lipid after tryptic digestion of proteoliposomes. The infrared amide I′ band, between 1600 and 1700 cm^{-1}, is mainly due to the stretching of the –C O group of the peptide bonds and provides information on the protein secondary structure. Band components of the acquired spectra can be evidenced by Fourier self-deconvolution (FSD) and second derivative, and can be assigned to particular secondary structures as described below. Band fitting to the original spectrum (without FSD) yields the proportion of spectral components. It can be considered that they reflect the proportion of secondary structure components in the protein. The band fitting resulted in small shifts from the band position detected initially and the values reported are the final results of the procedure [29]. The infrared spectra in the 1800–1500 cm^{-1} range are shown in the lower traces in each panel of Fig. 1 (spectra at 25 °C and 68 °C for proteoliposome is shown in a and b, and the same temperatures for the trypsinized sample are shown in c and d). Fourier self-deconvolution (FSD) of the spectra using a resolution increase factor $k=2$ [29] is also shown in Fig. 1. We also analyzed the spectral shape as a function of temperature. We recorded the spectra in the 22–72 °C temperature range, subtracted the baseline between 1600 and 1700 cm^{-1} and normalized the spectral area (Fig. 2a for proteoliposome and 2b for trypsinized proteoliposome). The differences in absorbance at different wavenumbers as a function of the temperature were plotted in Fig. 2c and d.

For proteoliposomes containing the whole protein at 25 °C we identified band components at 1620 and 1636 cm^{-1} corresponding to β-strands, at 1655 cm^{-1} corresponding to α-helix, at 1669 cm^{-1} assigned to β turns, and at 1679 cm^{-1} assigned to turns (see Table 1). New remarkable features appeared above 50 °C: the bands at 1616 and 1684 cm^{-1}. These bands have been clearly assigned to intermolecular contacts that result from the aggregation of unfolded segments [30,31]. It must be noted that bands representative of α-helix and β-sheet remained at a high temperature. Thus, we have concluded that some segments of the ATPase keep their secondary structure while others participate in intra-molecular, self-aggregation at a high temperature. A comparison of columns 3 and 6 in Table 1 shows that the spectra at 68 °C displayed a larger proportion of unordered chains (new band at 1641 cm^{-1}), a decrease in the amount of β-strand (1630 cm^{-1}) and α-helix structures (bands at 1652 cm^{-1}) as compared with the spectra at 25 °C (bands at 1620 plus 1636 and 1655 cm^{-1}). The difference spectra in Fig. 2a and c also show that the temperature increase produced a decrease in the central region of the spectra corresponding to β-strands and α-helix (for example, the absorbance differences measured at 1655 cm^{-1}) together with an increase in the unfolded, self-aggregated structures (absorbance differences measured at 1616 and 1684 cm^{-1}). Fig. 2c shows that these spectral changes occurred in a sigmoidal way within a broad temperature range, beginning at 40 °C with a midpoint for the transition at 50–53 °C suggesting a transition between folded and unfolded states although with low cooperativity. Considering that further self-aggregation was not observed above 60 °C and the sigmoidal shape of the transition, we can assume that a homogeneous state was reached at a high temperature. In this case, the proportion of spectral components in Table 1 should represent the proportion of secondary structure and unfolded-aggregated segments in each protein molecule. Still, we cannot ascertain at this point the topological organization of aggregated proteins within the lipid membrane. We do not know, for example, if aggregated complexes are organized in small or large patches or the number of protein molecules involved in an aggregated complex.

The fraction of protein that remained bound to the lipids after the proteolytic digestion displayed a remarkably different infrared spectrum as compared to the whole protein spectrum. FSD and further band fitting detected component bands at 1626 cm^{-1} (β-strands), 1652 cm^{-1} (α-helix) and 1640 cm^{-1} and 1664 cm^{-1} (unordered structures). The band at 1664 cm^{-1} was not originally detected by FSD and it was arbitrarily added to obtain an acceptable fitting. We remark upon two main differences in the trypsinized preparation as compared to the whole protein. On one side we

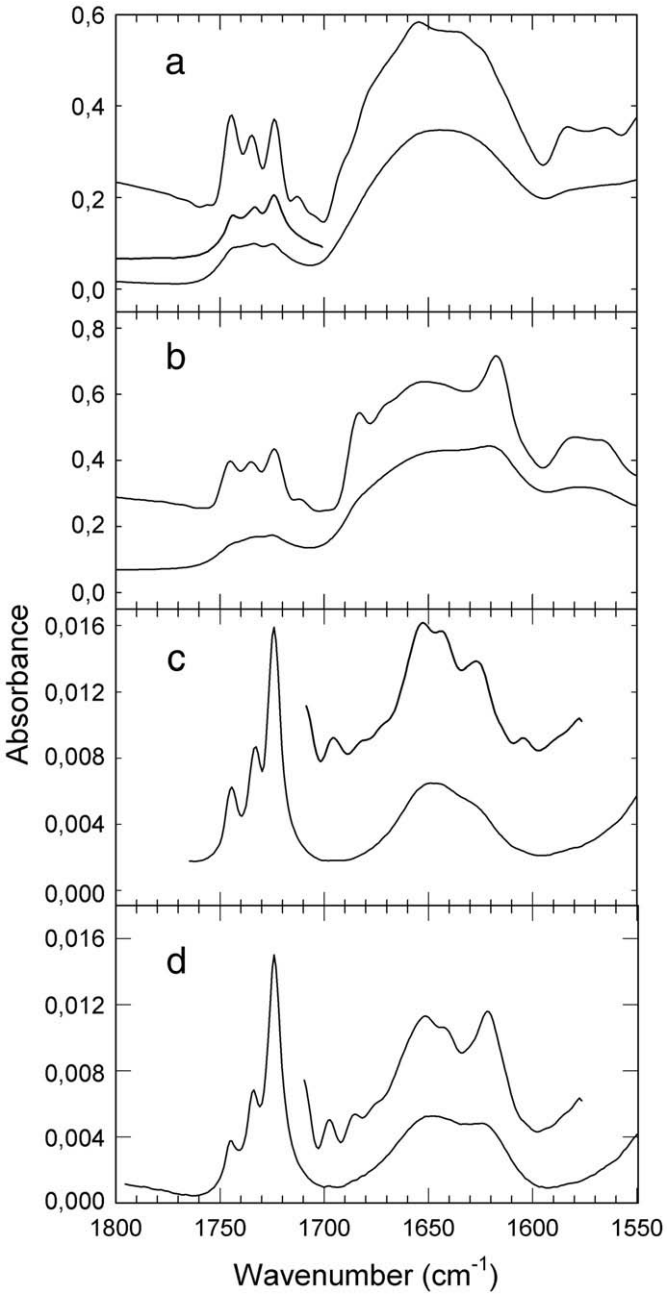

Fig. 1. Proteoliposomes infrared absorption spectra. Whole Na,K-ATPase reconstituted in liposome: 25 °C (a) and 68 °C (b). Trypsinized proteoliposomes: 25 °C (c) and 68 °C (d). In each panel, the lower trace corresponds to the direct, not deconvolved spectra and upper traces are the FSD using a $k = 2$ factor, and FWHH of 18 cm^{-1}. The middle trace in panel A is the absorption band, not deconvolved, of the pure lipid. Aqueous media contained D_2O with KCl 150 mM.

observed a relative decrease in the bands corresponding to β-strands: in the whole protein bands at 1620 plus 1636 cm^{-1} corresponding to β structure account for 45% of the amide I′ area (25 °C, Table 1), while the trypsinized proteoliposome displayed a band at 1626 cm^{-1} with 27% of the area (25 °C, Table 2). The second difference was the effect of increasing temperature. The difference spectra also showed the appearance of a band centered below 1620 cm^{-1} with increasing temperature, which could be interpreted as an aggregated structure. Nevertheless, FSD and band fitting indicated that this band was actually the same band present in the low-temperature spectra. It corresponds to an increase in β-strands and it is not due to the aggregation of unfolded chains. This proposal is also sustained by the fact that we have not detected the appearance of the characteristic band at 1684 cm^{-1} at high temperatures in the trypsinized material. The spectral changes occurred in a continuous way above 70 °C as shown in Fig. 2d. No sigmoidal shape was detected for the transition within the temperature range studied. If a cooperative transition was present, the midpoint should have been above the midpoint of the transitions observed in the whole protein.

Several authors described that the Na,K-ATPase denaturation process above 50 °C leads to an α–α form aggregation, without the participation of the β-subunit, and that heating above 60 °C leads to a complete loss of the enzymatic activity [21,25]. The infrared spectra of trypsinized proteoliposome are in agreement, since the aggregation bands were not present, indicating that the aggregation occurs through extra-liposomal α-subunit domains.

Besides this, we have demonstrated in a previous work that the Na, K-ATPase, both for the solubilized enzyme and the proteoliposome, lost its activity without considerable loss of the secondary structure [25].

Esmann et al. [32] have studied the structural characteristics of Na, K-ATPase from shark rectal glands by extensive trypsinization and also reviewed in the literature the spatial organization of the trypsinized protein, both in the membrane-bound form and in the detergent-solubilized state. An important point in interpreting the data obtained is that the trypsin proteolysis of the α- and β-subunits is dependent on the sodium or potassium concentrations used, resulting in different residual proteins with different aggregation capacities [32,33].

3.2. Effect of protein on the lipid organization

Infrared absorption bands from the different chemical groups of phospholipids yield information about the structure of the lipid bilayer and on protein–lipid interactions. The position of bands around 2920 and 2850 cm^{-1} due to symmetric and antisymmetric stretching of $-CH_2-$ groups in the acyl chains is dependent on the lipids phase state [34], while the band at 1750–1700 cm^{-1} due to vibration of –C O group of glycerol in the phospholipid provides information on interactions in the polar head group region [35]. Fig. 3 shows the band position due to the symmetric stretching of $-CH_2-$ as a function of the temperature: for a lipid preparation in the absence of protein, for a proteoliposome prepared with the whole ATPase, and for a trypsinized proteoliposome. In the samples prepared in the absence of protein, the band was displaced from 2917 cm^{-1} at a low temperature to 2922 cm^{-1} at higher temperatures, indicating a transition from an ordered gel to a chain-disordered liquid-crystalline phase. The transition occurred with a midpoint at about 55 °C and over a temperature range broader than that expected for pure PC or PE. When the whole ATPase was present in the bilayer, the lipid phase transition occurred at a lower temperature, at about 35 °C, and with a lower degree of cooperativity. In the gel phase, below 35 °C, the band was at higher wavenumbers as compared with the band in the absence of protein, indicating that the lipid was more disordered in the presence of the protein. The shift of the lipid phase transition to a lower temperature indicated that the interaction of the ATPase was more favorable with the lipid in the liquid-crystalline phase [35]. In the absence of the cytosolic domain, the new system (consisting of lipids and the transmembrane region) organized itself in a different way: within the temperature range in which the pure lipid is in the gel phase, lipids associated with the transmembrane region become more disordered according to the position of the infrared band. Increasing the temperature produced a slight and rather continuous shift to higher wavenumbers without the presence of a cooperative phase transition. At temperatures at which the pure lipid is in the liquid-crystalline phase, in the trypsinized proteoliposome, the lipid was more ordered than the pure lipid at temperatures corresponding to the liquid phase.

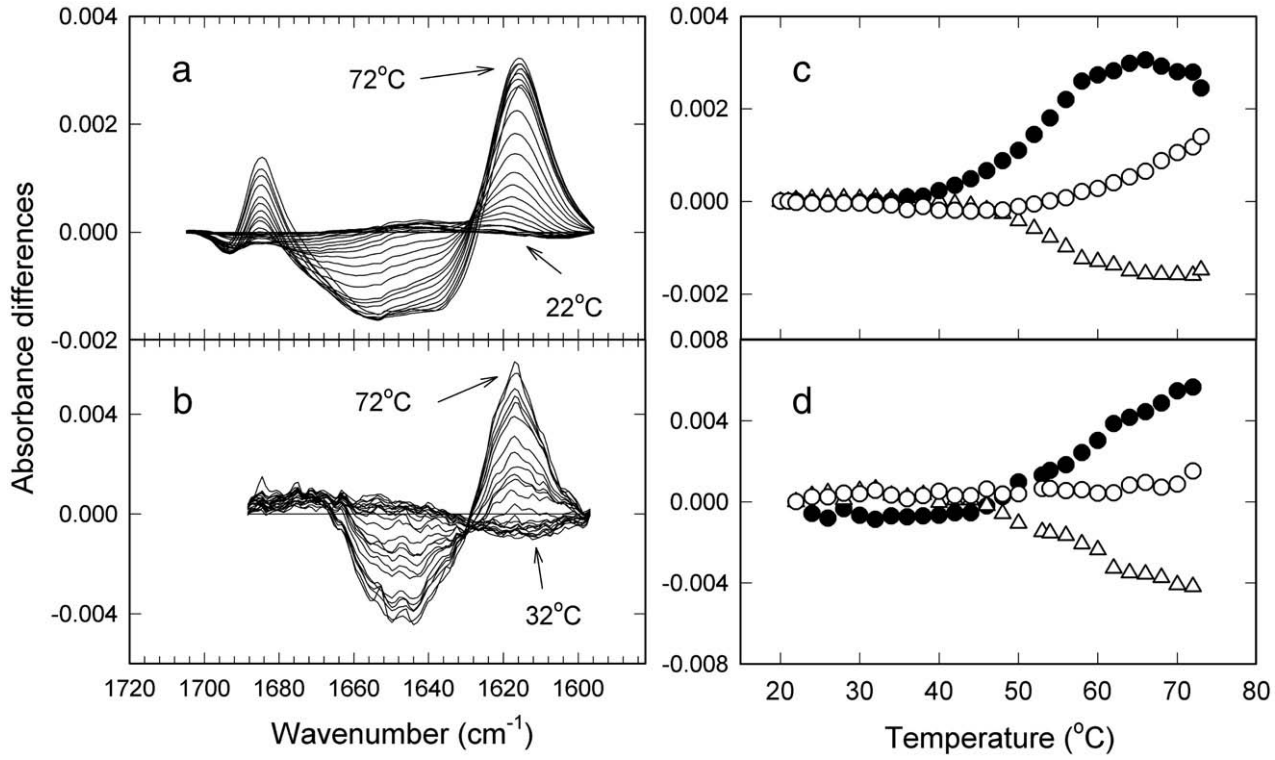

Fig. 2. Spectra difference of the amide I′ band at different proteoliposome temperatures prepared with the whole Na,K-ATPase (a) and trypsinized proteoliposome (b). Differences were obtained by subtracting the spectra at 20 °C from the spectra at different temperatures. The temperature intervals between the spectra were about 2 °C. Lipid composition and aqueous medium were as in Fig. 1. For panel c, data were taken from a; for panel d, data were taken from b. (●) 1616 cm^{-1}; (○) 1684 cm^{-1}; (△) 1655 cm^{-1}.

The activity of membrane proteins can be regulated by several physical properties of the lipid membrane including composition, phase state and length of the hydrocarbon chains. Theoretical models explain this regulation considering the thermodynamics of the interactions of lipid hydrocarbon chains with the hydrophobic transmembrane segment of the protein [36] and the heterogeneous profile of lateral pressure exerted by the membrane on the transmembrane segment [37]. As described by these models, mutual interactions between the protein and the lipids produce both of the following: shifts of the temperature of the lipid phase transition and shifts in the equilibrium between different conformations (activities) of the protein [38]. The observation that Na,K-ATPase shifts the lipid phase transition suggests the existence of this kind of mutual interactions and that the biological activity could be regulated by lipid properties within the natural membranes in the liquid-crystalline phase.

Table 1
Proteoliposomes infrared absorption data at 25 °C and 68 °C.

25 °C			68 °C		
Band position (cm^{-1})	FWHH (cm^{-1})	Amide I′ band (%)	Band position (cm^{-1})	FWHH (cm^{-1})	Amide I′ band (%)
			1616	25	23
1620	32	29	1630	27	19
1636	24	16			
			1641	20	13
1655	28	35	1652	28	14
			1660	18	9
1669	21	6	1671	18	14
1679	21	12			
			1684	16	9

Table 2
Trypsinized proteoliposome infrared absorption data at 25 °C and 68 °C.

25 °C			68°		
Band position (cm^{-1})	FWHH (cm^{-1})	Amide I′ band (%)	Band position (cm^{-1})	FWHH (cm^{-1})	Amide I′ band (%)
1626	23	27	1621	22	31
1640	23	26	1639	22	25
1652	22	31	1653	22	29
1664	24	15	1668	25	15

3.3. Tryptophan fluorescence

We studied the changes in the microenvironment of Trp residues as a function of the temperature by fluorescence spectroscopy [39].

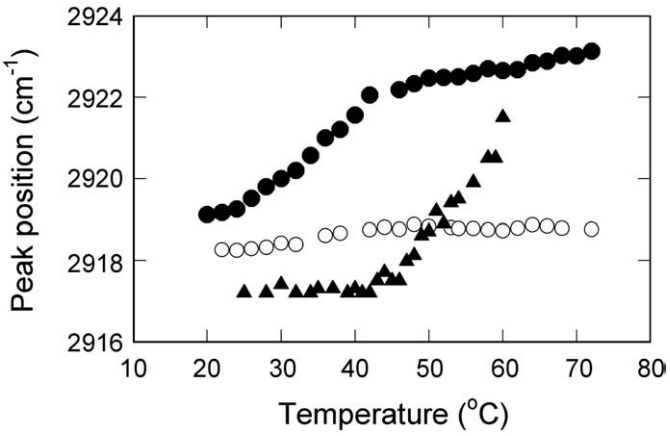

Fig. 3. Band position corresponding to the symmetric stretching of the $-CH_2-$ group of phospholipids: (▲) pure lipid processed in the absence of protein; (●) proteoliposome prepared with the whole Na,K-ATPase; (○) trypsinized proteoliposome.

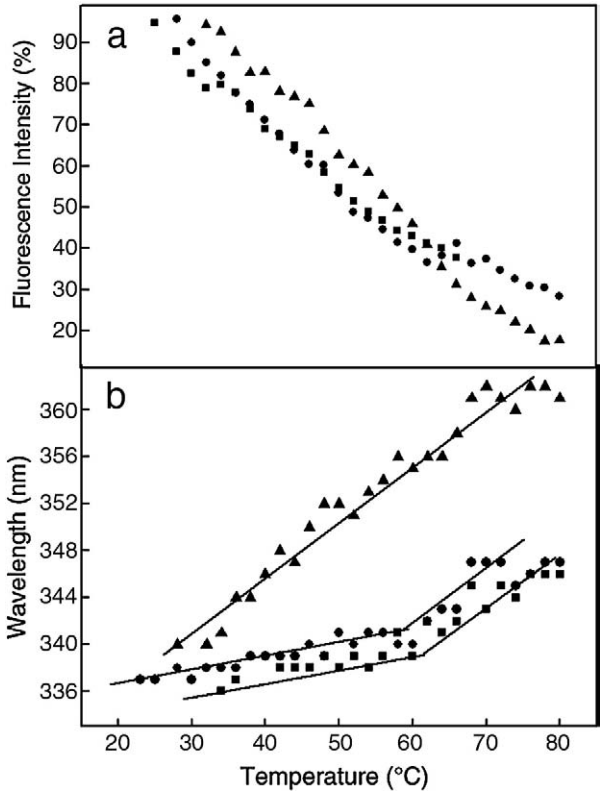

Fig. 4. Fluorescence intensity (a) and position of the emission spectra maximum (b) of: (■) solubilized Na,K-ATPase equilibrated in buffer Tris–HCl 5 mM with 150 mM KCl; (●) proteoliposome; and (▲) trypsinized proteoliposome.

Fluorescence intensities decreased with increasing temperature in a rather continuous way, without remarkable slope changes, in the three samples (Fig. 4a). Most probably, these trends are due to the natural decrease of fluorescence with temperature rather than reflecting conformational changes in the proteins. On the other hand, the dependence on the temperature of the emission band position revealed clear differences and structural changes in the different preparations. Both curves, for the solubilized and reconstituted protein, showed a noticeable change in the slope at 60 °C (Fig. 4b). Instead, for the trypsinized sample, a continuous red shift with an increase in temperature was observed.

There are 12 Trp residues in the sequence of Na,K-ATPase: 4 are located in the transmembrane segments, 4 in the extracellular domains, and 4 in the intracellular domains [40]. At this point we cannot discriminate the contribution of these Trp residues to the final emission spectrum, but the trend is that they were globally more exposed to the aqueous medium as the temperature increased as indicated by the red shifts of the spectra. The slope change at 60 °C in the curves is in agreement with a cooperative unfolding at this temperature. It must be emphasized that the increase of Trp exposure occurs together with protein aggregation as detected by FTIR, suggesting that the aggregation of Na,K-ATPase was not a massive, unspecific precipitation, but rather a specific interaction. Cooperative transition occurred only for the whole protein, whether solubilized or reconstituted, but not in the fraction remaining after trypsinization. The remaining tryptophan residues in this fraction continuously increased their exposure to the aqueous environment indicating that they are not involved in local cooperative conformational changes within the temperature range studied.

The enzyme is initially in the $(\alpha\beta)_2$ form, and can lose the activity when subunit separation occurs [3]. This triggers the aggregation process through the α-subunits, according to what was revealed by the infrared spectroscopy. Besides that, kinetic studies strongly suggest that, in the DPPC:DPPE-liposome systems, the Na,K-ATPase is found in the same $(\alpha\beta)_2$ form. The latter observation derives from a comparative study on the solubilized enzyme, in which it was found that the effect of ions (Na^+ and K^+) and the substrates ATP and pNPP showed the same behavior [3,4]. Therefore, according to our present results, we propose that a same mechanism may be occurring in both $C_{12}E_8$-solubilized Na,K-ATPase and that reconstituted in DPPC:DPPE-liposomes.

FTIR and fluorescence results show that with temperature increase, at 50/60 °C, the enzyme loses its activity and aggregation process occurs, but without a complete loss of its secondary and tertiary structures (~30%).

Using different forms of Na,K-ATPase (solubilized enzyme, DPPC:DPPE-liposome reconstituted without or with trypsinization), it is possible to deduce some important points: (i) the physical state of the lipids was different for the different systems; (ii) when the cytoplasmic domain is removed by trypsin treatment, the lipids acquire a structure that is more disorganized than in the gel phase of pure lipid, but more organized than the lipid in the liquid phase; (iii) the aggregation process is highly dependent on the cytoplasmatic domain.

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