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Topology of sarcoplasmic reticulum Ca^{2+}-ATPase: An infrared study of thermal denaturation and limited proteolysis

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Abstract

Sarcoplasmic reticulum Ca^{2+}-ATPase structure and organization in the membrane has been studied by infrared spectroscopy by decomposition of the amide I band. Besides the component bands assignable to secondary structure elements such as α-helix, β-sheet, etc. . . . , two unusual bands, one at 1,645 cm^{-1} in H_2O buffer and the other at 1,625 cm^{-1} in D_2O buffer are present. By perturbing the protein using temperature and limited proteolysis, the band at 1,645 cm^{-1} is tentatively assigned to α-helical segments located in the cytoplasmic domain and coupled to β-sheet structure, whereas the band at 1,625 cm^{-1} arises probably from monomer–monomer contacts in the native oligomeric protein. The secondary structure obtained is 33% α-helical segments in the transmembrane plus stalk domain; 20% α-helix and 22% β-sheet in the cytoplasmic domain plus 19% turns and 6% unordered structure. Thermal unfolding of Ca^{2+}-ATPase is a complex process that cannot be described as a two-state denaturation. The results obtained are compatible with the idea that the protein is an oligomer at room temperature. The loss of the 1,625 cm^{-1} band upon heating would be consistent with a disruption of the oligomers in a process that later gives rise to aggregates (appearance of the 1,618 cm^{-1} band). This picture would also be compatible with early results suggesting that processes governing Ca^{2+} accumulation and ATPase activity are uncoupled at temperatures above 37 °C, so that while ATPase activity proceeds at high rates, Ca^{2+} accumulation is inhibited.

Keywords: Ca^{2+}-ATPase; infrared spectroscopy; protein structure; proteolysis; sarcoplasmic reticulum; thermal analysis

Knowledge of the structure–function relationship is essential in understanding the molecular mechanisms underlying the membrane-controlled biological processes. The X-ray three-dimensional structure of membrane proteins is still not well known, except for a few cases. Therefore, other lower resolution spectroscopic methods have to be used to gain insight of the structure and function of membrane proteins. Sarcoplasmic reticulum Ca^{2+}-ATPase is an integral membrane protein that pumps calcium out of the cytoplasm during striated muscle relaxation (Martonosi, 1996). This ATPase is part of a family of P-type ion pumps that includes several cation-activated ATPases having in common ten predicted transmembrane helical segments (Stokes et al., 1994).

The structure of sarcoplasmic reticulum Ca^{2+}-ATPase has been predicted from the amino acid sequence (MacLennan et al., 1985) and from electron microscopy observations (Toyoshima et al., 1993). The protein appears to consist of an extensive beak-shaped cytoplasmic domain containing interconnected α-helical and β-strand segments, a stalk connecting the beak with the membrane, and the ten transmembrane segments characteristic of P-type ion pumps (Stokes et al., 1994). The cytoplasmic domain contains the active sites of ATP hydrolysis and phosphorylation while the Ca^{2+} channel is expected to be associated to the transmembrane domain. Some Ca^{2+}-binding sites, probably related to Ca^{2+} translocation, which is in turn coupled to ATP hydrolysis, can also be found in the cytoplasmic and lumenal regions of the membrane close to the transmembrane helices. The available data do not permit the precise localization of functional sites. Different experimental approaches, such as susceptibility of protein domains to protease digestion or changes in the interaction between protomers to form oligomeric structures, have been undertaken in order to understand the structural changes that link protein phosphorylation with cation translocation. Also, the quaternary structure of the pump is still undefined. Although it is generally accepted that the ATPase molecules form dimers and/or higher oligomers, monomeric Ca^{2+}-

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ATPase molecules are capable of performing all the steps of the reaction cycle (Andersen, 1989).

Heat is a convenient perturbing agent that will alter not only proteins, but also the lipid moiety of membranes. Thermally-induced perturbations of sarcoplasmic reticulum have been characterized by measuring ATPase activity and calcium uptake. It was found that between 37 and 40 °C the processes governing Ca^{2+} accumulation and ATPase activity were uncoupled (Inesi et al., 1973). According to differential scanning calorimetry (DSC), the thermal unfolding of Ca^{2+}-ATPase from sarcoplasmic reticulum takes place between 47 and 55 °C (Lepock et al., 1994). This thermal denaturation is a complex irreversible process showing an exothermic peak at slightly higher temperatures than the main unfolding denaturation peak. However, in monomeric Ca^{2+}-ATPase, thermal unfolding can be described with only one endothermic peak (Merino et al., 1994).

Proteolysis of membrane proteins has been used to characterize the transmembrane segments or different folding domains. P-type ion pumps are of particular interest because of the presence of large cytoplasmic and transmembrane domains that must accommodate the pathways for ion translocation. Proteolysis of Ca^{2+}-ATPase has been well characterized using different proteinases such as trypsin (Stewart et al., 1976; MacLennan et al., 1985), proteinase K (Juul et al., 1995), or a combination of both enzymes (Corbalán-García et al., 1994). In all cases, digestion is sequential, and an extensive treatment leaves only the transmembrane segments and the lumenal part.

Infrared spectroscopy (IR) has been extensively used to characterize membrane proteins because the presence of lipids does not interfere with the measurements (Arrondo & Goñi, 1993). IR has been used with Ca^{2+}-ATPase to characterize the $E_1 \rightarrow E_2$ conformational transition either by studying the amide I band (Arrondo et al., 1987; Villalaín et al., 1989) or by using caged compounds (Buchet et al., 1991; Barth et al., 1994). Recently, the kinetic mechanism of the enzyme has been described by time-resolved difference spectroscopy using ATP (Barth et al., 1996). Also, IR has been used to characterize the proteolytic digestion of the nicotinic acetylcholine receptor (Görne-Tschelnokow et al., 1994) and P-type ATPases such as the *Neurospora* H^+-ATPase (Vigneron et al., 1995) or the Ca^{2+}-ATPase from sarcoplasmic reticulum (Corbalán-García et al., 1994).

In the present work, IR is used to study the structure of the Ca^{2+}-ATPase from sarcoplasmic reticulum by decomposing the amide I band, arising mainly from the C=O stretching vibrations of the peptidic bond, and characterizing each of the component bands obtained. Temperature and limited proteolysis have been used to further characterize the amide I band components, to explore the protein oligomeric structure, and to explore the interactions between different segments of the polypeptide.

Results

Secondary structure of Ca^{2+}-ATPase

The infrared amide I band, produced mainly by the C=O stretching vibrations of the peptidic bond, is conformationally sensitive. Differences in dihedral angles and hydrogen bonding among the different protein conformations give rise to a composite band enclosing the structural information of the protein (Susi, 1969). This information can be extracted using mathematical techniques. First, band narrowing procedures allow the determination of the number and position of the compounds, and then iteration procedures are used to band-fit the original envelope. The use of spectra measured in both H_2O and D_2O buffers help in assigning to specific protein features the bands obtained (Arrondo et al., 1993). Figure 1 shows the decomposed amide I envelope of Ca^{2+}-ATPase from sarcoplasmic reticulum in H_2O and D_2O. The spectral parameters corresponding to the various components are listed in Table 1. Band assignment is not yet a straightforward procedure because of the sensitivity of infrared spectroscopy, and a stepwise method must be followed (Arrondo et al., 1994; Bañuelos et al., 1995); first the assignment of well-defined bands is accomplished, then the bands presenting any problems are discussed. In H_2O buffer six bands are seen attributable to protein backbone structure; the band at 1,658 cm^{-1} comprises the α-helix plus the overlapping unordered structure bands. The bands at 1,632 and 1,693 cm^{-1} are due to β-sheet and those at 1,672 and 1,684 cm^{-1} arise from β-turns. The band around 1,645 cm^{-1} in H_2O has not a precise assignment yet. It was not described using homopolypeptides or a set of soluble proteins (Susi, 1969), but recently it has been found in several proteins and attributed to loops or bends of the protein (Fabian et al., 1992; Echabe et al., 1995) or to coiled coils (Reisdorf & Krimm, 1996). In D_2O, the band at 1,657 cm^{-1} corresponds only to α-helix because the unordered structure component shifts

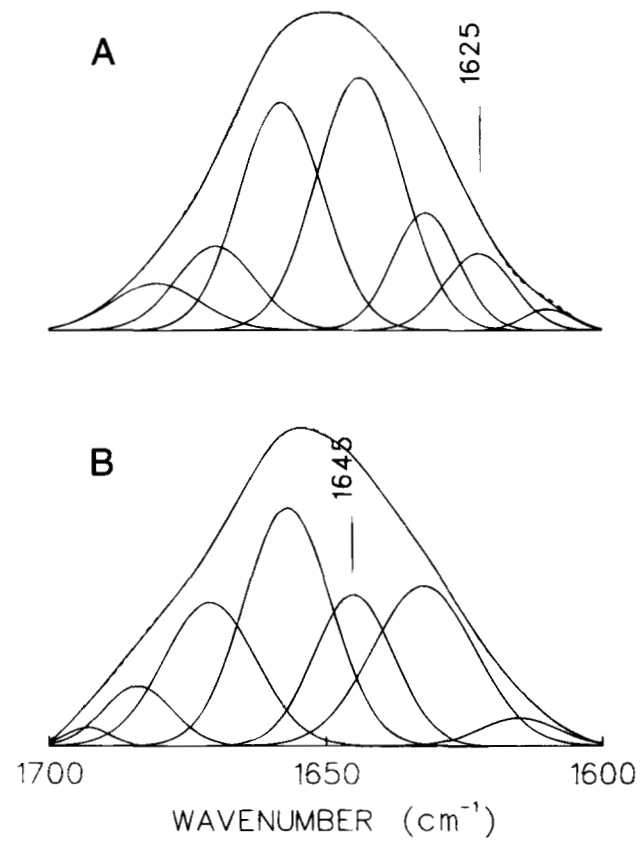

Fig. 1. Amide I band decomposition of Ca^{2+}-ATPase from sarcoplasmic reticulum in H_2O **(A)** and D_2O **(B)** buffer. The parameters corresponding to the component bands are reflected in Table 1. Contour: experimental, continuous line; reconstructed by the addition of band components, dashed line.

Table 1. *Amide I band decomposition parameters corresponding to Ca^{2+}-ATPase from sarcoplasmic reticulum in either H_2O or D_2O buffer*

H_2O			D_2O		
Position	% Area	Width	Position	% Area	Width
1,693	<1	9	1,680	5	14
1,684	9	13	1,669	9	15
1,672	10	16	1,657	33	21
1,658	39	21	1,644	29	18
1,645	20	18	1,632	19	15
1,632	22	24	1,625	6	14

to 1,643 cm^{-1}; the 1,632 cm^{-1} band corresponds to β-sheet and the 1,644 cm^{-1} to unordered structure plus the as yet unidentified component observed in H_2O. The bands at 1,669 and 1,680 cm^{-1} arise from β-turns and from the high frequency component $(0,\pi)$ of β-sheet. This contribution of β-sheet should be lower than 1%, according with the H_2O results. Also, a band that is not immediately assignable is observed in D_2O at 1,625 cm^{-1}. Bands at this low frequency were first observed in aggregated proteins and only in D_2O buffer (Yang et al., 1987). In nondenatured cytochrome *c* oxidase, a band at this frequency has been found and attributed to protein–protein contacts corresponding to the subunits of oligomeric proteins (Arrondo et al., 1994). Such a band was not observed in the monomeric protein (Echabe et al., 1995). It has also been seen in deuterated β-strands with intermolecular contacts (Susi, 1969).

Combining the values arising from the spectra in both H_2O and D_2O media and assuming that the molar absorptivities are alike for the different protein structures (as stated in Material and methods), the percentage of assignable Ca^{2+}-ATPase secondary structure is: 33% α-helix corresponding to the band at 1,657 cm^{-1} in D_2O, 6% unordered that is the difference between the D_2O and the H_2O bands, 22% β-sheet, and 19% β-turns. The bands at 1,645 cm^{-1} in H_2O and at 1,625 cm^{-1} in D_2O that are not easily assignable will be discussed later in light of the thermal and proteolytic changes induced in the protein.

Thermal denaturation of Ca^{2+}-ATPase

Heat produces in many proteins an irreversible denaturation that is characterized in the infrared spectrum in D_2O buffer by the appearance of two bands, located at around 1,620 and 1,680 cm^{-1}, indicative of protein aggregation (Arrondo et al., 1994). Figure 2 shows a three-dimensional plot of the deconvolved spectra of sarcoplasmic reticulum ATPase in the 20–75 °C range. It is apparent that the thermal profile does not correspond to a typical native → denatured two-state process. Instead, intermediate species appear to be present between ≈45 and 55 °C, different from both the native and the denatured states. A more detailed information can be obtained by plotting the different band positions of the amide I components (Fig. 3). Each component has a different thermal profile. Thus, the plot of band position vs. temperature of the com-

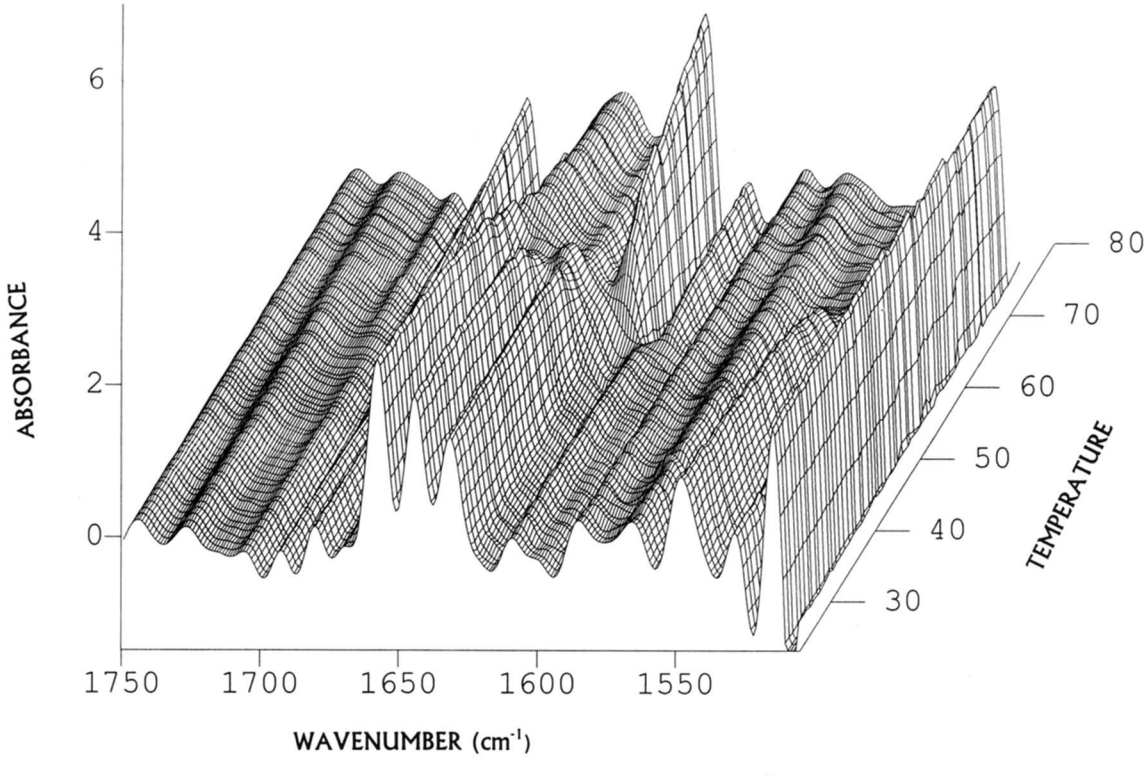

Fig. 2. A three-dimensional plot showing the effect of heating on the IR spectrum of Ca^{2+}-ATPase. The spectra were acquired in a D_2O medium, pD 7.4. Fourier self-deconvolution was performed using a resolution-enhancement factor of 3 and a Lorentzian line shape of 18 cm^{-1}.

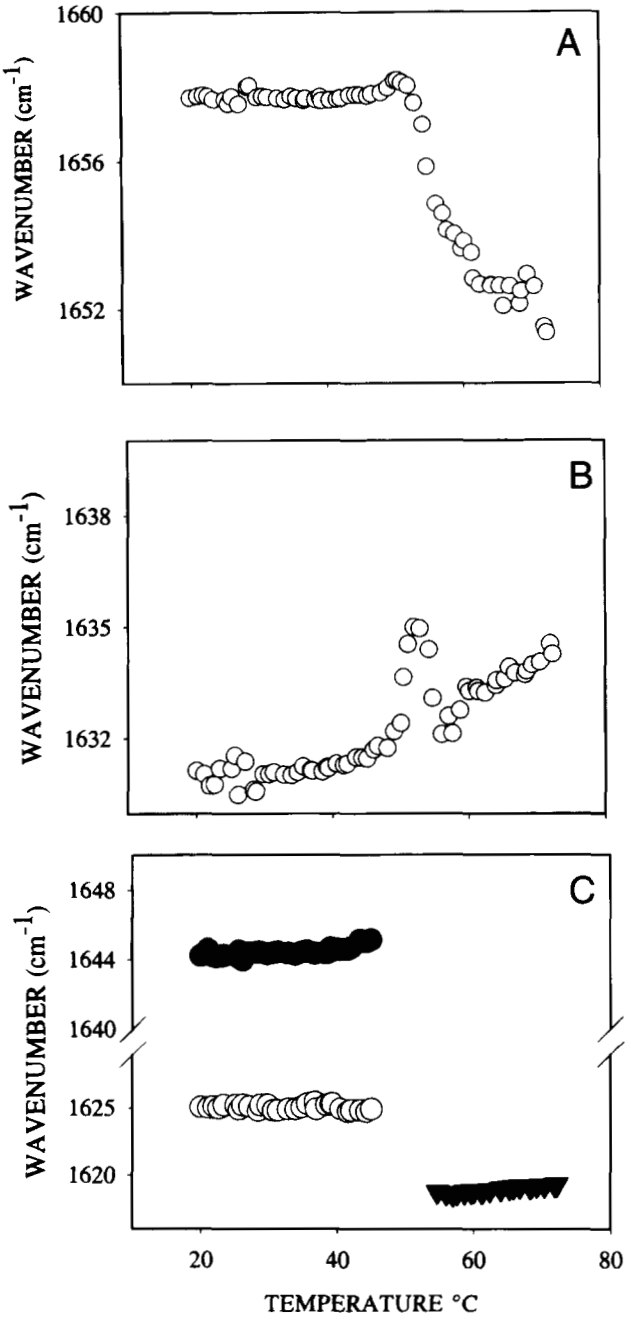

Fig. 3. Thermal profiles of the amide I band components at 1,657 cm^{-1} (α-helix), 1,632 cm^{-1} (β-sheet), 1,644 cm^{-1}, and 1,625 cm^{-1} measured in a D_2O medium.

ponent around 1,657 cm^{-1} and corresponding to the α-helix (Fig. 3A) is closer to a typical two-state denaturation, sigmoid with a midpoint around 55 °C, very similar to the one obtained for mitochondrial cytochrome *c* oxidase (Arrondo et al., 1994). The downshift in wavenumber of the α-helical component is found in both H_2O and D_2O media in many proteins (Arrondo et al., 1994). This downshift (smaller in H_2O than in D_2O buffers) has been interpreted as the result of changes in helix conformation, together with the full deuteration of the protein. A similar plot corresponding to the β-sheet structure (Fig. 3B) shows a more complex profile, with a shift in band position from 45 to 52 °C, then shifting back between 52 and 56 °C. Figure 3C shows how the bands located around 1,644 and 1,625 cm^{-1} disappear at 45 °C, while the band around 1,618 cm^{-1} corresponding to the temperature-induced aggregation does not appear until 55 °C, in agreement with the pattern observed with the β-sheet band. It must be noted that the band at 1,625 cm^{-1} is assigned to protein–protein contacts in the native, active protein, whereas the band at 1,618 cm^{-1} is indicative of aggregation. The change in conformation along protein denaturation is depicted in the corresponding decomposed amide I bands (Fig. 4). The spectrum at 52 °C shows the 1,655 cm^{-1} band as the major component (58%) together with a band at 1,636 cm^{-1} (36%) that includes not only the β-sheet structure, but can also overlap with the unordered structure component (Martínez et al., 1996). Note that the bandwidths of the various components at this temperature are increased. Bandwidths are related to librational motions of the chemical groups (Casal et al., 1980; Cortijo et al., 1982) and increases in bandwidth have been also observed in intermediate states of *Paracoccus denitrificans* cytochrome oxidase (Echabe et al., 1995). At 75 °C the major component is un-

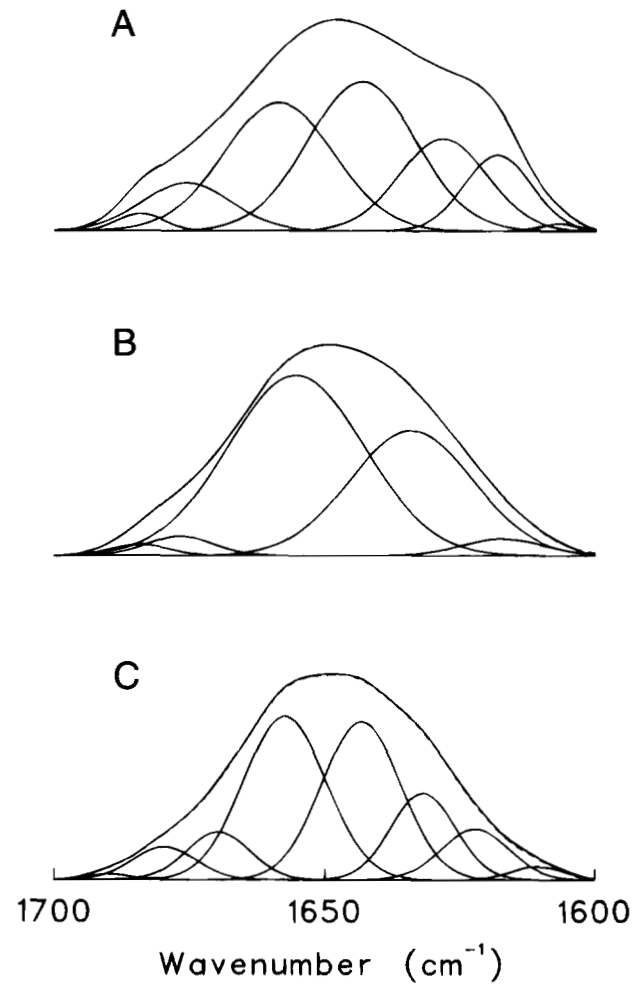

Fig. 4. Amide I band decomposition of sarcoplasmic reticulum Ca^{2+}-ATPase in the native state (**A**) at 25 °C, intermediate state (**B**) at 52 °C and denatured (**C**) state at 73 °C produced by temperature and measured in a D_2O medium.

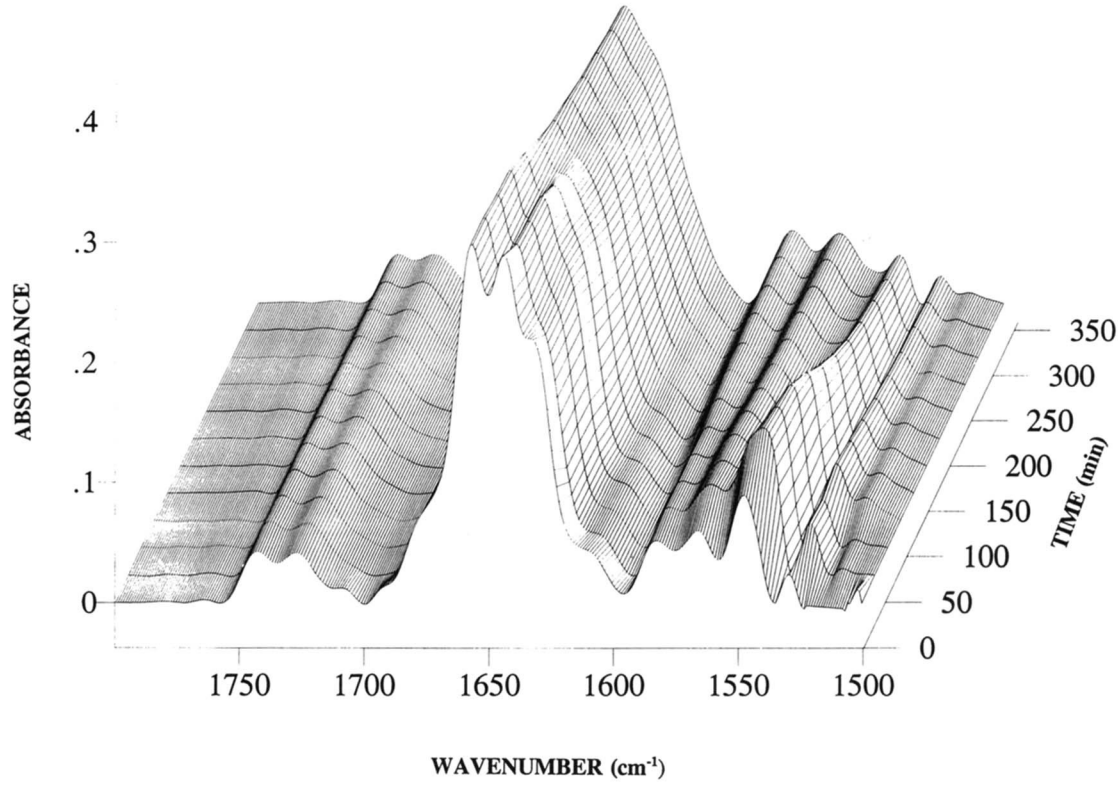

Fig. 5. A three-dimensional plot of deconvolved spectra with a resolution enhancement factor of 2 and a Lorentzian lineshape of 18 cm^{-1} of the Ca^{2+}-ATPase amide I band measured in a D_2O medium. The spectra are measured in the presence of 1:50 molar ratio of trypsin at intervals of 30 min at the temperatures indicated in Table 2.

ordered structure (30%) together with extended structures represented by the component at 1,627 cm^{-1} (25%) attributable to some distorted β-sheet, plus the bands corresponding to aggregation or intermolecular protein contacts (8%).

Proteolysis

Sarcoplasmic reticulum was subjected to proteolysis either with trypsin or with a cocktail composed of trypsin, chymotrypsin, and proteinase K. The aim of the experiment was to relate the loss in activity produced by the proteases with changes in the amide I band. To avoid uncontrolled proteolysis, the reaction was started at low temperature and was increased in several steps until the ATPase activity was completely lost. Incubation of sarcoplasmic reticulum produced in either case, trypsin or cocktail, a change in the amide I band shape concomitant with a loss of the bands at 1,625 cm^{-1} and at 1,669 cm^{-1} (Fig. 5). Table 2 shows the activity of the protein together with the amide I area at different times and

Table 2. *Effect of trypsin on Ca^{2+}-ATPase activity and structure at different incubation times and temperatures*[a]

Incubation time/temperature (°C)	Activity (%)	Total area (%)	1,657 cm^{-1} α-helix	1,631 cm^{-1} β-sheet	1,670 + 1,680 cm^{-1} turns	1,644 cm^{-1}[b]
0/4	100	100	1.0	1.0	1.0	1.0
30/13	130	95	1.2	1.0	0.6	1.03
60/17	68	93	1.25	1.0	0.5	0.86
90/23	54	90	1.3	1.0	0.55	0.86
120/37	20	88	1.4	0.9	0.4	0.86
150/37	3	86	1.5	0.9	0.35	0.80
180/37	0	85	1.6	0.8	0.3	—

[a]The activity is expressed as a percentage of the initial activity. The structures are expressed as ratios with respect to the native structures. The band at 1,625 cm^{-1} present in the native structure is lost after 30 min incubation.

[b]The assignment of this band is discussed in the text and attributed to an α-helix coupled with β-sheet. The band at 1,644 cm^{-1} disappears from this spectrum after 150 min.

temperatures after incubation with trypsin. Activity gradually decreases and becomes zero after about 150 min. The decrease in the area of the amide I band gives an idea of the loss of peptide bonds. In the first 30 min, ≈5% of the area is lost, but an increase in activity is produced concomitant with an increment in α-helical content and a reduction in turns. Further treatment with trypsin decreases by an additional 10% the total area of the amide I before the activity is lost; the decrease in activity is accompanied by an increase in the relative amount of α-helix and a decrease in turns with little or no change in β-sheet. Once the activity is lost, the band at 1,644 cm^{-1} is no longer present and a further increase in the α-helical content is produced. A more drastic treatment with the protease cocktail (not shown) shows a qualitatively similar behavior although with slight quantitative differences, since protease combination attacks the protein at more points than trypsin alone.

Discussion

Structure of the sarcoplasmic reticulum Ca^{2+}-ATPase

Infrared spectroscopy has been used in the present work to elucidate different aspects of sarcoplasmic reticulum Ca^{2+}-ATPase structure and organization in the membrane. The structural basis for the study is the 14 Å resolution picture obtained by cryoelectron microscopy (Toyoshima et al., 1993) and the structural model deduced from the sequences of several P-type ATPases together with other available data (Stokes et al., 1994). The transmembrane domain has 10 helical segments, whereas the cytoplasmic domain possesses α-helix and β-sheet structure. Decomposition of the amide I band envelope shows that 33% of its area corresponds to the 1,657 cm^{-1} component, undoubtedly assigned to α-helix. This percentage would account for around 350 amino acids being involved in canonical α-helix structure. In turn, this could correspond to the transmembrane α-helical segments and the stalk, but it would not take into account the α-helical segments that according to the current view of the ATPase structure occur in the cytoplasmic domain. A previous infrared study based on partial least-squares analysis and a set of proteins with known structures, predicted 48% of α-helix and 20% of β-sheet (Corbalán-García et al., 1994). The discrepancy can be due to the sensitivity of individual infrared band components to changes in hydrogen bonding or dihedral angles, or to the possibility of coupling between structural segments of the protein (Reisdorf & Krimm, 1996). A not easily assignable component in the Ca^{2+}-ATPase amide I is the band at 1,644 cm^{-1} in H_2O. In cytochrome b_5, this band had been assigned to a 3_{10}-helix (Holloway & Mantsch, 1989) because of a distortion of the dihedral angles. However, it is still under debate whether the 3_{10}-helix band appears at this position, or else around 1,660 cm^{-1}, depending on the environment. More often, a band at this position has been assigned to flexible loops (Fabian et al., 1992) because of the intermediate position between the coiled helix and the extended sheet. Recently, the study of coiled coils has shown that coupled structures such as α-helical bundles can give rise to bands shifted from the frequencies of the canonical structures (Reisdorf & Krimm, 1996). In the ATPase structure, the band at 1,645 cm^{-1} represents as much as a 20% of the protein backbone and this makes very difficult its assignment to a distorted structure such as 3_{10}-helix. The assignment of this band to flexible loops would be possible in a picture where the cytoplasmic domain would consist of β-sheet strands connected by long loops. Besides, a coupling between α-helix and β-sheet could be considered. Up to now, α-helix $\rightarrow$ α-helix coupling has been described in coiled coils and proposed for helical bundles (Reisdorf & Krimm, 1996), but no infrared evidence has been found for $\alpha \rightarrow \beta$ coupling even if such interaction has been postulated as possible (Cohen & Parry, 1986).

We have altered the Ca^{2+}-ATPase structure by temperature and proteolysis. In both cases, changes in the infrared spectrum (band position and/or band area) in the region around 1,632 cm^{-1} are concomitant with a loss of the band at 1,644 cm^{-1} and an increase in the band at 1,657 cm^{-1}, assigned to α-helical content (Table 2). This relationship, together with the above observations, supports the idea that the band at 1,645 cm^{-1} in the H_2O spectrum of Ca^{2+}-ATPase corresponds to cytoplasmic α-helical segments coupled to β-sheet structure. Then, the protein structure would consist of 33% α-helical segments in the transmembrane plus the stalk domain, 20% α-helix and 22% β-sheet in the cytoplasmic domain plus 19% turns and 6% unordered structure.

Temperature effect on Ca^{2+}-ATPase

The complex thermal denaturation of Ca^{2+}-ATPase is not only characterized by the loss of the band at 1,644 cm^{-1} concomitant with the change in position of the 1,632 cm^{-1} band, also the band at 1,625 cm^{-1} in D_2O disappears at the beginning of the unfolding process. This band is different from the one at 1,618 cm^{-1}, indicative of aggregation, that appears around 55 °C. Bands at such low frequencies are not easily found in nondenatured proteins and have recently been assigned to protein–protein contacts in oligomeric proteins (Martínez et al., 1996). Thus, the thermal profile of this band would indicate that while the protein is oligomeric at room temperature, it becomes transiently a monomer in the process of thermal denaturation. DSC of sarcoplasmic reticulum also showed a complex profile that was attributed to the unfolding of two different domains of Ca^{2+}-ATPase (Lepock et al., 1994). Later, it was shown, also by DSC, that in monomeric Ca^{2+}-ATPase the thermal unfolding shows a single endothermic peak (Merino et al., 1994). These results would support a picture according to which the protein is an oligomer at room temperature, that through a transient monomeric form (loss of the 1,625 cm^{-1} band) proceeds toward aggregation (onset of the 1,618 cm^{-1} band). This multistep denaturation pathway would also be consistent with early results by Inesi et al. (1973), in which it was found that between 37 and 40 °C the processes governing Ca^{2+} accumulation through ATPase activity are uncoupled, so that while ATPase activity proceeds at high rates, Ca^{2+} accumulation is inhibited. The oligomer–monomer transition would imply loss of Ca^{2+} uptake while the ATPase would remain active as long as the protein does not become aggregated. The temperature at which the putative monomerization occurs is similar to the ones found in previous DSC (Lepock et al., 1994) and infrared (Corbalán-García et al., 1994) work and is dependent on the way the sample is heated, a situation similar to the one observed for cytochrome *c* oxidase (Echabe & Arrondo, 1995). Native sarcoplasmic reticulum has a similar thermal denaturation profile (data not shown) only shifted 3–5 °C toward higher temperatures. The assignment of the band at 1,625 cm^{-1} to monomer–monomer contacts in nondenatured oligomeric proteins is also supported by previous studies of cytochrome oxidase, where the band is present in the oligomeric mitochondrial enzyme (Arrondo et al., 1994) but not in the monomeric form from *P. denitrificans* (Haltia et al., 1994), and by the recent data on tyrosine hydroxilase where a correlation has been found between the presence of the

1,625 cm^{-1} band and the monomer–monomer interaction (Martínez et al., 1996).

The proteolytic attack of Ca^{2+}-ATPase by trypsin is well characterized (Corbalán-García et al., 1994) so that it may be used for studying the structural changes related to activity loss. Trypsin attacks the protein in the cytoplasmic domain, first at Arg 505 and then at Arg 198. The changes associated with proteolysis affect β-turns and the oligomer $\rightarrow$ monomer transition. The rearrangement produced would not affect the $\alpha \rightarrow \beta$ coupling signaled by the band at 1,644 cm^{-1} until the activity is lost, and the uncoupling has occurred. Also interesting is the fact that some activity is still present well after protein digestion has begun either with trypsin or with the cocktail including proteinase K (not shown). This would indicate that some structures, probably loops or turns, can be removed without total loss of activity. This result would be compatible with the ability to retain calcium binding after cleavage of the polypeptide chain in the N-terminal region by proteinase K (Juul et al., 1995).

Materials and methods

Preparation of sarcoplasmic reticulum vesicles and purified Ca^{2+}-ATPase vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit back and leg muscle as described previously (Nakamura et al., 1976; Prado et al., 1983). Ca^{2+}-ATPase was purified using deoxycholate by method 2 of Meissner et al. (1973), yielding closed vesicles essentially free of extrinsic proteins, in which the Ca^{2+}-ATPase accounts for more than 90% of the total protein. Protein concentration was determined by the method of Lowry et al. (1951). Ca^{2+}-ATPase activity was assayed as previously described with an ATPase-regenerating system (Prado et al., 1983).

Proteolysis of sarcoplasmic reticulum Ca^{2+}-ATPase

Trypsin digestion was performed in parallel in the infrared cell and in a test tube. The samples for activity and electrophoresis were taken from the test tube at the same time each spectrum was acquired. The trypsin stock solution was added in a 1:50 molar ratio to sarcoplasmic reticulum at a final concentration of 25 mg/mL in a TES-sucrose buffer pH 7.4 containing 0.25 M sucrose, 0.1 M KCl, and 0.05 M TES. The samples were incubated at increasing temperatures every 30 min until reaching 37 °C. These temperatures allowed us the measurement of infrared spectra with the sample still active. Every 30 min, two 15 μL samples were taken from the incubation test tube and the digestion stopped by the addition of 10 μL PMSF solution (1,200 μg in 10 mL DMSO); one of the samples was used for activity measurements and the other one for electrophoresis. The electrophoresis was performed according to the method of Laemmli (1970) using 10% gels containing 0.8% bisacrylamide. The results are equivalent to those obtained previously by Corbalán-García et al. (1994).

Infrared spectroscopy

Infrared spectra of aqueous lipoprotein vesicle suspensions were obtained as described previously (Arrondo et al., 1987). The D_2O samples showed a band at 1,545 cm^{-1} indicating that the exchange was not complete, as in other membrane proteins (Earnest et al., 1990; Goormaghtigh et al., 1994). Prior to the measurements, the samples were left to stabilize until the residual amide II band absorbance was invariant with time. Since incubating these samples in D_2O buffer for as long as several hours did not change this residual amide II band, it can be assumed that all the accessible groups, including those of the lumenal side (Goormaghtigh et al., 1994), are exchanged. In order to estimate the amount of nonexchanged amide protons, the amide II band was decomposed, and the area of the 1,547 + 1,530 components (corresponding to the structural bands) was ratioed against the amide I area or the Tyr component at 1,515 cm^{-1} in both H_2O and D_2O, and these ratios were compared. In our sample, the H_2O/D_2O ratio is 7.28:1, implying that only around 12% of the amide protons were nonexchanged. The spectra were recorded in a Nicolet Magna 550 spectrometer equipped with a MCT detector using a demountable liquid cell (Harrick Scientific, Ossining, New York) with calcium fluoride windows and 6 μm spacers for samples in H_2O medium or 50 μm spacers for samples in D_2O medium. A tungsten–copper thermocouple was placed directly onto the window and the cell placed into a thermostated cell mount. Typically 1,000 scans for each, background and sample, were collected and the spectra obtained with a nominal resolution of 2 cm^{-1}. Data treatment and band decomposition of the original amide I have been described elsewhere (Castresana et al., 1988; Arrondo et al., 1989; Arrondo et al., 1993; Arrondo et al., 1994; Bañuelos et al., 1995). The mathematical solution to the decomposition may not be unique, but if restrictions are imposed such as the maintenance of the initial band positions in an interval of ± 1 cm^{-1}, the preservation of the bandwidth within the expected limits or the agreement with theoretical boundaries or predictions, the result becomes, in practice, unique. The results were verified by constructing artificial curves with the parameters obtained and adding those in order to reconstruct the amide I envelope. Comparison with the experimentally obtained original band showed that both original and reconstructed bands were identical. After obtaining the area of each component, the corresponding percentages were obtained assuming that the molar absorptivities for the different protein structures are the same. This assumption is under debate (De Jongh et al., 1996), but it has been proved to be correct by other workers (Byler & Susi, 1986; Echabe et al., 1995; Arrondo et al., 1996).

Thermal studies were carried out by a continuous heating method. This method uses the Series and Rapid Scan software from OMNIC (Nicolet Corp., Madison, Wisconsin). One hundred sixty-four interferograms/min, using 50 μm pathlength, are collected at 2 cm^{-1} resolution and averaged after each minute. The sample is heated in the interval 20–80 °C at 1 °C/min. Hence, each spectrum corresponds roughly to 1°. Temperature is monitored by a probe located at the edge of the window. After data recording, the interferograms are processed by ratioing with respect to a background, and spectra are obtained. The use of H_2O and D_2O spectra helps to improve the band assignment with respect to spectra taken in a single medium, but the error increases from 1–2% for spectra in only one medium up to around 4–5% for the present method (measured as the dispersion of values obtained in different sets of spectra).

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