Extensive Proteolytic Digestion of the (Ca^{2+}+Mg^{2+})-ATPase from Sarcoplasmic Reticulum Leads to a Highly Hydrophobic Proteinaceous Residue with a Mainly α-Helical Structure†

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ABSTRACT: The purified (Ca^{2+}+Mg^{2+})-ATPase from sarcoplasmic reticulum was subjected to extensive proteolysis by using trypsin and proteinase K. This digestion led to the elimination of a considerable portion of the protein, so that the lipid to protein weight ratio was increased from 0.44 in the purified ATPase to 1.20 after extensive proteolysis. After the digestion, the residue was found to be considerably enriched in hydrophobic amino acids. FT-IR spectroscopic studies indicated that the secondary structure of the proteolytic residue was enriched in α-helix with 75%, compared with 48% in the intact purified ATPase. FT-IR studies using ATR polarization showed that the α-helical part of the residue of proteolytic digestion was considerably more polarized than the purified ATPase, indicating that, on average, the α-helices of the residual protein should lie with an orientation closer to the normal to the plane of the membrane. Thermal denaturation studies showed that the residue of proteolysis was considerably more stable than the intact purified ATPase. This would be compatible with the residue being protected from denaturation by its hydrophobic location within the membrane. This study is experimental evidence of the α-helical structure of the membrane part of this protein, as suggested by predictions made from its known primary structure (Brandl et al., 1986).

The structure of membrane proteins is one of the main topics which remain elusive to detailed analysis. Except for very few exceptions, they have not been studied using high-resolution techniques like X-ray diffraction or nuclear magnetic resonance. Therefore, other alternative techniques of less resolution capacity are being used nowadays. The (Ca^{2+}+Mg^{2+})-ATPase from sarcoplasmic reticulum is a very representative integral membrane protein which relaxes striated muscle by pumping calcium out of the cytoplasm into the sarcoplasmic reticulum against a large concentration gradient (Bigelow & Inesi, 1992). The approaches used to study its structure are illustrative of the technology developed for membrane proteins in general. These include predictions made from the amino acid sequence and spectroscopic and diffraction studies [see Bigelow and Inesi (1992) for a recent review].

The predictions made from the cDNA-deduced amino acid sequence of the (Ca^{2+}+Mg^{2+})-ATPase from sarcoplasmic reticulum (MacLennan & Brandl, 1985; Brandl et al., 1986) have led to the supposition that the (Ca^{2+}+Mg^{2+})-ATPase would consist of two parts: a large cytoplasmic domain and a membrane domain. The portion located in the cytoplasm would be formed by 3 domains of β-structure linked between them and to the membrane by α-helical and random elements, whereas the portion of the protein situated inside the membrane would consist of 10 α-helices spanning the bilayer.

Other morphological studies, such as those made through electron microscopy (Molnar et al., 1990; Stokes & Green, 1990; Toyoshima et al., 1993), are not of sufficiently good resolution to confirm the secondary structure predictions mentioned above.

In this work, we have taken the approach of extensively digesting the purified (Ca^{2+}+Mg^{2+})-ATPase by using proteases, with the aim of eliminating the part of the protein which lies outside of the lipid bilayer on the cytoplasmic surface. This is assumed to be feasible since the intramembrane portion will be protected from the catalytic action of the proteases. Thereafter, we have utilized Fourier transform infrared spectroscopy (FT-IR),[1] which is a particularly effective method for probing and obtaining the secondary structure of a protein (Byler & Susi, 1986; Susi & Byler, 1986; Surewicz & Mantsch, 1988; Bandekar, 1992; Haris & Chapman, 1992). We have also combined the use of the attenuated reflection technique (ATR) with FT-IR in order to obtain information on the orientation of the protein in the membrane (Fringeli & Günthard, 1981). The polarized infrared spectra provide information on the mean orientation of the transition dipole moment of the amide, the side chain groups of the proteins, and the acyl chain of the phospholipids, and at the same time, the order parameter of the characteristic vibrational bands is evaluated by measuring the dichroic ratio (Fringeli & Günthard, 1981). This technique has been used previously to obtain information on the orientation of proteins and peptides in membranes, as, for example, melittin (Frey & Tamm, 1991; Weaver et al., 1992), (Na^++K^+)-ATPase (Buchet et al., 1991; Fringeli et al., 1989), δ-hemolysin (Bramer et al., 1987), bacteriorhodopsin (Yang et al., 1987), Pf1-phage protein (Azpiazu et al., 1993), and several synthetic peptides (Ishiguro et al., 1993; Goormaghtigh et al., 1991a) where

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[1] Abbreviations: ATR, attenuated total reflectance; FT-IR, Fourier transform-infrared spectroscopy; MOPS, 4-morpholinepropanesulfonic acid; PLS, partial least squares; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T-ATPase, trypsin-digested (Ca^{2+}+Mg^{2+})-ATPase; TP-ATPase, trypsin–proteinase K digested (Ca^{2+}+Mg^{2+})-ATPase.

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various orientations for the α-helix in the membrane have been reported, i.e., perpendicular plane arrangement as well as parallel and random orientation. Our results reveal, in agreement with the predictions mentioned above, that the membrane integral portion of this enzyme is mainly α-helical.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum Vesicles and Purified (Ca^{2+}+Mg^{2+})-ATPase Vesicles. Sarcoplasmic reticulum vesicles were prepared according to the method of Eletr and Inesi (1972). (Ca^{2+}+Mg^{2+})-ATPase was purified using deoxycholate by method 2 of Meissner et al. (1973), giving place to closed vesicles essentially free of extrinsic proteins, and where the (Ca^{2+}+Mg^{2+})-ATPase accounts for more than 90% of the total protein (Meissner et al., 1973). This preparation will be called purified ATPase throughout this work. Protein concentration was determined by the method of Lowry et al. (1951). Lipid concentration was estimated as previously described (Böttcher et al., 1961).

(Ca^{2+}+Mg^{2+})-ATPase Modification with Proteases. Trypsin-digested ATPase was obtained by mixturing purified ATPase (1 mg/mL) in 20 mM MOPS, pH 6.8, 80 mM KCl, and 1 M glycerol with 0.235 mg/mL trypsin for 30 min at 35 °C. The reaction was stopped by adding 0.47 mg/mL soybean trypsin inhibitor. Afterwards, the vesicles were centrifuged and washed twice at 100000*g* for 75 min at 4 °C in 20 mM MOPS, pH 6.8, and 80 mM KCl. After this, the pellet was resuspended in 10 mM MOPS, pH 7.0, and 0.3 M sucrose. This fraction will be abbreviated as T-ATPase. Where stated, a further digestion with proteinase K was performed to the trypsin-digested ATPase by incubating 1 mg/mL tryptically modified (Ca^{2+}+Mg^{2+})-ATPase, i.e., T-ATPase, in 10 mM Tris-HCl, pH 7.4, with 0.06 mg/mL proteinase K for 30 min at 25 °C. The reaction was stopped by adding 0.5 mM phenylmethanesulfonyl fluoride. This fraction will be abbreviated as TP-ATPase. The membrane fraction was centrifuged and resuspended as before for the trypsin-digested protein.

Amino Acid Composition Analysis. Three different samples were analyzed: (i) purified (Ca^{2+}+Mg^{2+})-ATPase; (ii) T-ATPase; and (iii) TP-ATPase. Hydrolysis was carried out in 2 mL of 6 N HCl and 0.2% 2-mercaptoethanol with 0.25 mg of protein and 0.01 mg of *N*-Leu as inner standard for 96 h at 110 °C in sealed tubes at vacuum. Then, the lipids were removed by two consecutive extractions with chloroform before lyophilization (chloroform/sample, 1:2 v/v). Amino acid derivatization with phenyl isothiocyanate was carried out by the method described by Bergman et al. (1986). Briefly, 40 μL of 99.5% ethanol/water/triethylamine (2:2:1, by volume) was added to each tube of the hydrolyzed samples and dried under vacuum. Samples were then derivatized by addition of 3 μL of 50% ethanol and subsequent addition of 7 μL of 99.5% ethanol/triethylamine/phenyl isothiocyanate (7:2:1, by volume). The reaction was allowed to proceed for 15–30 min at room temperature. Excess reagent was removed by high vacuum at room temperature overnight in a desiccator with solid NaOH. The phenylthiocarbamyl-amino acid determination was performed by high-performance liquid chromatography in a Beckman 114M as described by Heinrikson and Meredith (1984), using a Spherisorb ODS-2 column (25 × 0.46, 5 μm).

SDS-PAGE of (Ca^{2+}+Mg^{2+})-ATPase. SDS–PAGE gels containing 14% acrylamide in the running gel and 4% acrylamide in the stacking gel were made according to the method of Laemmli (1970). The gels were stained with silver using a Bio-Rad Silver Stain Plus Kit.

FT-IR Measurements. (Ca^{2+}+Mg^{2+})-ATPase, T-ATPase, and TP-ATPase were washed 2 more times with 20 mM MOPS/80 mM KCl pH 6.8 H_2O buffer for FT-IR measurements. Samples containing 0.4 mg of purified (Ca^{2+}+Mg^{2+})-ATPase, T-ATPase, or TP-ATPase were transferred to a Specac 20710 cell and placed in between two CaF_2 windows separated by 6-μm Teflon spacers (from Specac, Kent, U.K.). This spacer kept the absorbance of the H_2O band at 1640 cm^{-1} below 1, permitting quantitative spectral subtraction of the background (Dousseau & Pèzolet, 1990). FT-IR spectra were obtained in a Philips PU9800 Fourier transform infrared spectrometer equipped with a deuterated triglycine sulfate detector. Each spectrum was obtained by collecting 500 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using the sample shuttle accessory in order to average background spectra between sample spectra over the same time period. The spectrometer was continuously purged with dry air in order to remove atmospheric water vapor from the bands of interest. The samples were equilibrated at 25 °C for 25 min before acquisition. For temperature studies, samples were scanned between 25 and 80 °C at 5 °C intervals with a 2-min delay between each consecutive scan with a water bath interfaced to the spectrometer computer.

FT-IR Data Analysis. Subtraction from buffers taken at the same temperatures as the samples was performed interactively using Spectra-Calc (Galactic Industries, Salem, MA). The criterion used for subtraction was removal of the band near 2125 cm^{-1} and a flat baseline between 1770 and 2100 cm^{-1}. Frequencies at the center of gravity were measured by taking the top 10 points of each specific band and fitting them to a Gaussian function.

Quantitative analysis of the secondary structure of a range of soluble and membrane proteins was made by a partial least-squares (PLS) method using the PLSplus program version 2.1 (Galactic Industries). A full mathematical description of this method has been given previously (Haaland & Thomas, 1988). The FT-IR spectra of 25 proteins were used to create the calibration set, taking into account the secondary structure content estimated from X-ray diffraction. The proteins used were alcohol dehydrogenase, carbonic anhydrase, concanavalin A, cytochrome *c*, chymotrypsin, chymotrypsinogen, hemoglobin, insulin, lactic dehydrogenase, lysozyme, myoglobin, papain, pepsin A, pepsinogen, ribonuclease S, ribonuclease A, subtilisin, superoxide dismutase, thermolysin, and trypsin [see Dousseau and Pèzolet (1990) and Lee et al. (1990) for a thorough discussion of the method used to determine the protein secondary structure by infrared spectroscopy]. Moreover, some membrane peptides were used in the training set. These were bombesin, colicin A, colicin E, and the M13 and Pf1 coat proteins. The FT-IR spectra of these peptides were obtained from the originally published spectra (Azpiazu et al., 1993; Goormaghtigh et al., 1991b; Rath et al., 1991; Sanders et al., 1993) or by collecting the FT-IR spectra as stated above (bombesin in egg yolk phosphatidylcholine, 35:1 lipid to protein molar ratio). Quantitative estimations of the secondary structure of these proteins were obtained from the following works: bombesin from Carver and Collins (1990); colicin A from Goormaghtigh et al. (1991b) and Parker et al. (1989); colicin E from Rath et al. (1991); Pf1 from Shon et al. (1991) and Turner and Weiner (1993); and M13 from Turner and Weiner (1993). The overall intensities of the absorbance spectra after subtraction were normalized to unity (Dousseau & Pèzolet, 1990) before analysis with the PLS method.

Band-narrowing strategies were applied in order to resolve the component bands in the amide I region. Fourier self-deconvolution (Kauppinen et al., 1981) of the subtracted spectra was carried out using a Lorentzian shape and a triangular apodization function with a resolution enhancement parameter, K, of 2.2, which is lower than log(signal/noise) [see Mantsch et al. (1988)], and a full width at half-height of 18 cm^{-1}. These parameters assumed that the spectra were not overdeconvolved as was evidenced by the absence of negative side lobes. The number of bands detected was the same as in the second derivative of the spectra.

For ATR measurements, 3 mg of purified (Ca^{2+}+Mg^{2+})-ATPase, T-ATPase, or TP-ATPase was oriented by depositing the samples on a ZnSe ATR crystal. These membrane protein films were dried in a N_2 atmosphere for 1 h under vacuum and rehydrated afterward (Buchet et al., 1991). ATR-IR spectra were obtained by using an infrared beam polarized with a KRS-5 polarizer (Specac) and a horizontal ATR holder (Spectra Tech, U.K.). The ATR crystal, made from ZnSe, was cut with an incident angle of 45°. Under these conditions, the infrared beam travels along the crystal plate about 35 total internal reflections before exiting, and then it is directed to the detector. Each spectrum was ratioed with a background spectrum obtained with the polarizer set at 0° ($A_\parallel$) or at 90° ($A_\perp$) using membrane-free ATR crystals. Evaluation of the dichroic ratio ($R^{ATR} = \Delta A_\parallel / \Delta A_\perp$) was estimated from the infrared ATR spectra. $\Delta A_\parallel$ and $\Delta A_\perp$ are the differences in reflection absorbance spectra between the ATR crystal covered with purified (Ca^{2+}+Mg^{2+})-ATPase, T-ATPase, or TP-ATPase and the clean ATR crystal measured with the polarized infrared light parallel ($\Delta A_\parallel$) and perpendicular ($\Delta A_\perp$) to the normal of the membrane protein film.

Chemicals. Proteinase K, MOPS, phenylmethanesulfonyl fluoride, and the proteins and peptides used to determine the secondary structure by FT-IR were purchased from Sigma, Madrid, Spain (except where noted). Phenyl isothiocyanate, amino acid standards, and triethylamine were obtained from Pierce, Oud-Beijerland, The Netherlands. Trypsin and soybean trypsin inhibitor were obtained from Boehringer, Barcelona, Spain. Acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, and Silver Stain Plus were purchased from Bio-Rad, Hercules, CA. All other reagents were purchased from Merck, Darmstadt, Germany.

RESULTS AND DISCUSSION

Characterization of the Product of Proteolysis of the (Ca^{2+}+Mg^{2+})-ATPase. It is well-known that upon mild conditions trypsin cleaves the protein at Arg505, yielding two fragments (A and B). Further digestion leads to the cleavage of fragment A at Arg198 into subfragments A_1 and A_2 (Stewart et al., 1976; MacLennan et al., 1985). However, extensive digestion with trypsin for 30 min at 35 °C resulted in the cleavage of fragments A_1, A_2, and B into smaller peptides which produce accumulation of a number of peptides with molecular mass smaller than 20 kDa, although faint protein bands of 24 kDa still remain after extensive digestion (see Figure 1, well II). Therefore, we used proteinase K to extend the proteolysis beyond trypsinolysis. Proteinase K after trypsin digestion has a small effect on the electrophoretic pattern; nevertheless, the band of ~24 kDa was fragmented, and there was an increase in the size of the band corresponding to ~20 kDa (Figure 1, well III).

It could be expected that the action of the proteases will result in the digestion of those parts of the protein situated

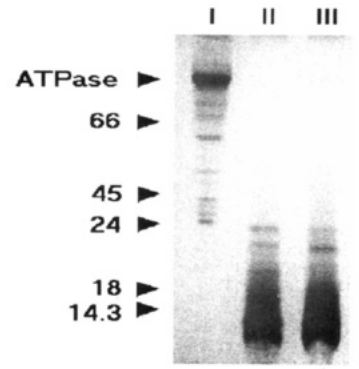

FIGURE 1: Polyacrylamide gel electrophoresis in SDS of purified ATPase (I), digested ATPase with trypsin (II), and digested ATPase with proteinase K after trypsin digestion (III). Molecular mass markers are indicated with arrows: bovine albumin (66 kDa), egg albumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

in the cytoplasmic side and outside of the membrane, since these parts would be easily accessible to the proteases. On the contrary, the intramembranous part of the protein and the part situated facing the lumen will be protected from the action of the enzyme. According to current proposed models (Clarke et al., 1989a,b) the action of trypsin in C-terminal peptide bonds of arginine and lysine residues would produce the intramembranous segments $F_{57}\rightarrow W_{77}$, $F_{88}\rightarrow V_{106}$, $K_{262}\rightarrow P_{282}$, $I_{298}\rightarrow C_{318}$, $L_{764}\rightarrow L_{781}$, $V_{790}\rightarrow G_{808}$, $W_{832}\rightarrow W_{854}$, $P_{896}\rightarrow S_{915}$, $N_{930}\rightarrow I_{947}$, and $D_{963}\rightarrow K_{985}$. We could also include the interconnecting loops located in the luminal side of the membrane, $F_{78}\rightarrow A_{87}$, $V_{283}\rightarrow K_{297}$, $G_{782}\rightarrow P_{789}$, $W_{855}\rightarrow E_{895}$, and $Y_{948}\rightarrow D_{962}$, and the loops located in the external side of the membrane, $S_{48}\rightarrow Q_{56}$, $W_{107}\rightarrow R_{110}$, $L_{253}\rightarrow S_{260}$, $L_{319}\rightarrow R_{324}$, Y_{765},$F_{809}\rightarrow R_{820}$, $E_{826}\rightarrow G_{831}$, $L_{916}\rightarrow R_{925}$, $M_{926}\rightarrow V_{929}$, and $F_{986}\rightarrow R_{989}$, accounting for 356 amino acid residues, which is 42% over the total weight of the enzyme. Further digestion with proteinase K would lead to extensive digestion of the external loops, because of its wide range of protease activity in several peptide bonds. Supposing that the intramembranous fragments and the interconnecting loops located in the luminal side of the membrane would persist in these conditions of digestion, these fragments would include 292 amino acids, which accounts for 34% over the total weight of the enzyme.

As a consequence of protein digestion, there was a considerable change in the lipid/protein ratio. Whereas purified (Ca^{2+}+Mg^{2+})-ATPase has a lipid/protein ratio of 0.44 ± 0.027 (w/w), T-ATPase has 0.92 ± 0.05 and TP-ATPase 1.20 ± 0.024 (these figures represent mean values ± standard deviations corresponding to five different experiments). Therefore, trypsin digestion produces an increase of 2.1 times in the lipid to protein ratio (w/w). For this case, a predicted value of 2.3 can be calculated. In the same way, the action of proteinase K produces an increase of 2.7 times in the lipid to protein ratio (w/w), the calculated value being 2.95. From these results, it is evident that the reduction in mass of the protein operated by these proteases closely agrees with the reduction predicted assuming the previously proposed models (Clarke et al., 1989a,b), and the action of proteases that we are suggesting here.

Amino acid analysis was performed on purified ATPase, T-ATPase, and TP-ATPase. These results are shown in Table 1, and it is immediately evident that the percentage of hydrophobic amino acids is increased upon digestion. For example, Ile was increased from 7.86% in ATPase to 9.96% in TP-ATPase, Leu increased from 11% in ATPase to 16.5% in TP-ATPase, and Phe increased from 4.36% in ATPase to

Table 1: Amino Acid Composition (%) of Purified ATPase and Digested ATPase[a]

amino acid	predicted values		experimental results		
	ATPase[b]	membrane[c]	ATPase	T-ATPase[d]	TP-ATPase[e]
Ala	8.98	10.90	9.00	8.74	7.28
Pro	4.64	5.09	4.73	5.46	6.00
Met	3.40	3.27	3.00	2.30	3.00
Val	8.47	9.82	9.61	9.56	10.10
Ile	7.64	10.54	7.86	9.93	9.96
Leu	9.40	16.00	11.00	16.60	16.50
Phe	3.82	5.82	4.36	6.67	7.76
Asx	9.00	6.18	8.00	5.65	6.50
Glx	10.53	7.63	9.00	8.47	9.76
Ser	5.78	4.00	5.10	4.52	3.65
Gly	6.92	7.27	6.80	8.87	7.45
Thr	6.81	5.09	6.87	3.42	1.45
Tyr	2.27	2.54	1.93	2.26	3.80
His	1.23	2.54	1.58	1.60	1.87
Arg	5.26	1.10	4.98	2.85	2.33
Lys	5.70	2.18	6.54	3.10	2.59

[a] The predicted values are also shown assuming that the model presented by Clarke et al. (1989) for the transmembrane portion is correct. [b] Amino acid composition of the $(Ca^{2+} + Mg^{2+})$-ATPase as deduced from cDNA analysis (Brandl et al., 1986). [c] Membrane-inserted part of the protein plus interconnecting luminal loops. [d] Digested protein using trypsin. [e] Digested protein using trypsin plus proteinase K.

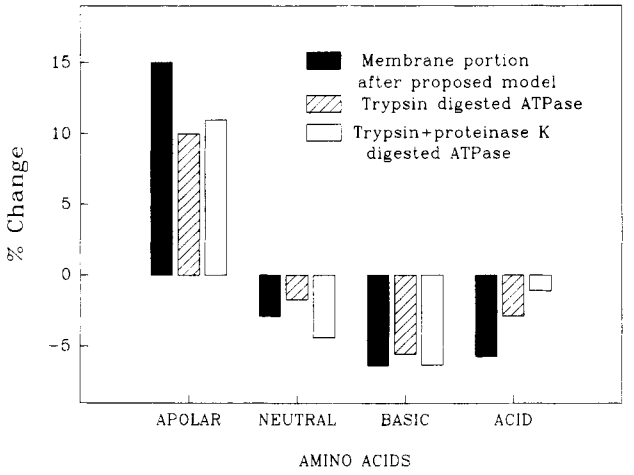

FIGURE 2: Change in amino acid composition (%) after extensive proteolytic digestion with trypsin (hatched bars) or with trypsin and proteinase K (white bars). The theoretical values for the transmembrane portion are also shown (black bars) after the model of Clarke et al. (198). The amino acids are grouped according to their polarity (Ala, Pro, Met, Val, Ile, Leu, and Phe are considered apolar amino acids; Ser, Gly, Thr, and Tyr are neutral amino acids; His, Arg, and Lys are basic amino acids; and Glu, Gln, Asp, and Asn are acidic amino acids).

7.76% in TP-ATPase. On the other hand, other polar amino acids decreased, for example, Asp, from 8.0% in ATPase to 6.15% in TP-ATPase, or Lys, which decreased from 6.54% in ATPase to 2.59% in TP-ATPase. Table 1 also shows the amino acid composition deduced from cDNA analysis (Brandl et al., 1986) and the composition of the membrane portion plus luminal connecting loops, calculated as stated above. Note that Cys and Trp were not determined, and Asn and Gln were analyzed as Asp and Glu, respectively. At the same time, fairly good agreement exists between the composition of the ATPase deduced from the cDNA sequence and that experimentally found, and there is also good agreement between the compositions of the membrane loop portion plus the luminal segments and the TP-ATPase. This is further illustrated in Figure 2, where amino acids are divided into apolars, neutrals, basics, and acids, showing good agreement between what should be expected from predicted structures and what was

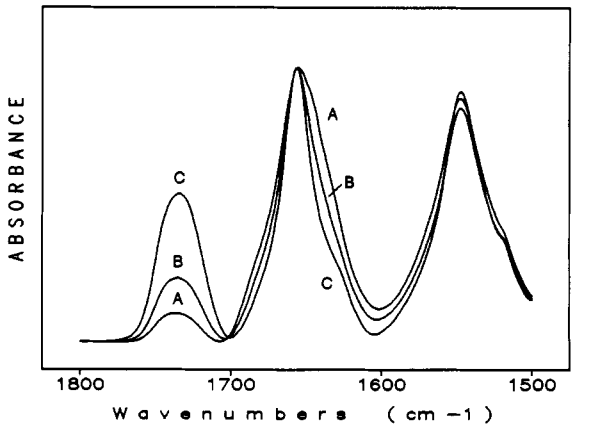

FIGURE 3: FT-IR spectra of (A) purified $(Ca^{2+}+Mg^{2+})$-ATPase, (B) T-ATPase, and (C) TP-ATPase. The infrared spectrum of H_2O buffer has been subtracted from the different protein spectra. The bandwidth of the amide I band, centered at 1655 cm^{-1}, significantly decreases upon digestion whereas the C=O vibration band of the phospholipids increases (see text for details). Spectra have been normalized.

observed for TP-ATPase, so that there was an increase in the percentages of apolar amino acids and a decrease in all the others.

Dumont et al. (1985) observed by extensive proteolysis of bacteriorhodopsin with proteinase K (24 h) that some transmembrane regions were digested. However, we have been using 30 min only for our proteolytic digestions, so that digestion of intramembranous portions of the protein is not so likely in our case. Although it is not possible to completely exclude that the digestion could have affected portions of the ATPase inside the membrane, our results show very close agreement with the predicted model, in which only digestion of the extramembranous portions is assumed (see above).

FT-IR Studies of Protein Secondary Structure. The most useful IR bands of a protein are the amide modes and among them, amides I and II. Amide I, primarily a C=O stretching mode, with small contributions from C–N stretching and C–N deformations, has been used to determine the secondary structure of proteins, soluble or membranous, either by resolving the underlying peaks of the amide I band contour and their assignment to different secondary structural components (Susi & Byler, 1987; Dong et al., 1990) or by partial least-squares and factor analysis methods (Lee et al., 1990; Dousseau & Pèzolet, 1990), where no curve-fitting and amide I band component assignments are necessary [see also Surewicz et al. (1993) for a recent review on the infrared method to estimate the quantitative secondary structure of proteins].

As was mentioned above, upon digestion of the protein, the lipid to protein ratio must increase, as has actually been found. Therefore, the IR bands corresponding to the phospholipid must increase upon digestion with respect to the protein bands. This is what has been found in the IR spectra of the digested ATPase. Figure 3 shows the FT-IR spectra of purified $(Ca^{2+}+Mg^{2+})$-ATPase, T-ATPase, and TP-ATPase. The spectral region plotted contains the lipid carbonyl C=O streching mode (1750–1710 cm^{-1}), the peptide bond amide I mode (1680–1620 cm^{-1}), and the peptide bond amide II mode (1560–1520 cm^{-1}). It can be observed in Figure 3, where the spectra have been normalized with respect to the intensity of the amide I band, that the area under the ester carbonyl band of the membrane phospholipids centered at 1730 cm^{-1} grows as a consequence of digestion. Hence, the ratio of the area under the C=O band to that of the amide I band increases upon digestion, indicating an increase in the lipid to protein

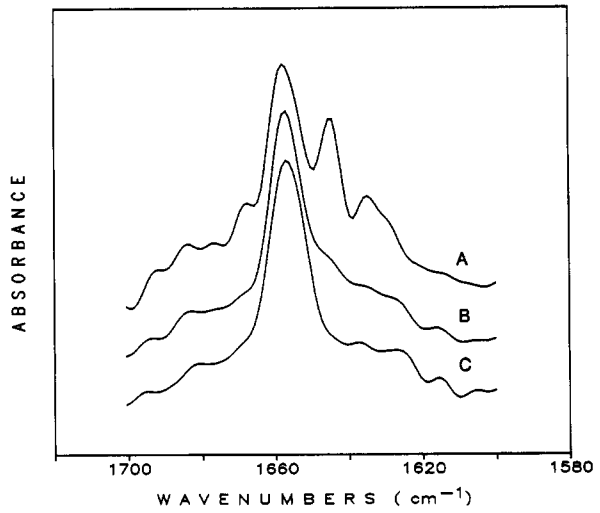

FIGURE 4: FT-IR deconvolution of (A) (Ca^{2+}+Mg^{2+})-ATPase, (B) T-ATPase, and (C) TP-ATPase, by using a bandwidth at half-height of 18 cm^{-1} and a resolution parameter, K, of 2.2.

ratio, in accordance with the chemical analysis of the lipid to protein ratio of these samples (see above). The maximum of the amide I band of the purified ATPase, as well as the digested ATPase, is located at 1656 cm^{-1}, in agreement with a previous work (Villalaín et al., 1989), indicating the predominance of α-helical structures in this protein. It is also very important to notice that the width of the amide I band decreases with digestion, so that T-ATPase is narrower than purified ATPase and TP-ATPase is still narrower than T-ATPase. The maximum of the band remains, however, at the same frequency, implying an enrichment in α-helical structure as a consequence of proteolytic digestion.

Spectral deconvolution allowed us to obtain the decomposition of the amide I band into its underlying components (Figure 4). In the deconvolved spectra, it can be observed that the intensity of the 1656 cm^{-1} band, i.e., α-helix, increases for the digested proteins compared with the purified ATPase, whereas the other bands, corresponding mainly to β-sheet and turns, remarkably decrease upon digestion.

An estimation of the secondary structure was performed using the PLS method, based on the known structures of a panel of proteins (Lee et al., 1990; Dousseau & Pèzolet, 1990). Recent reviews by Arrondo et al. (1993) and Surewizc et al. (1993) have thoroughly discussed the problems inherent to the different methods used for the estimation of the secondary structure of proteins by using FT-IR. It is known that the position of the amide I band of proteins can be affected by the environment and secondary structure. However, soluble proteins, of the globular type as those used by us as models, will have both hydrophilic and hydrophobic environments. Also the (Ca^{2+}+Mg^{2+})-ATPase has an extramembranous portion which will have hydrophobic environments as well. On the other hand, the residue obtained after proteolytic digestion will be constituted predominantly by intramembranous fragments, but it will contain also some extramembranous fragments, such as the luminal loops. Due to all these reasons, we find justified the use of soluble proteins in the training set of the PLS method as used in this work. In any case, we have completed the panel with some mainly intrinsic membrane proteins. Unfortunately, it is not possible to easily find many examples of membrane proteins of which the membrane structure is known by high-resolution methods such as NMR or X-ray diffraction. In our training set, we have included a total of five intrinsic proteins. It should be remembered that the PLS method will need, in order to work properly, a very wide training set, covering all possibilities of protein secondary structures (Dousseau & Pèzolet, 1990; Lee et al., 1990).

Table 2: Secondary Structure Components (%) from PLS-Analyzed Spectra of Purified and Digested (Ca^{2+} + Mg^{2+})-ATPase

(Ca^{2+} + Mg^{2+})-ATPase	α-helix	β-sheet	turns	random
purified	48	20	14	18
T-ATPase	62	9	9	20
TP-ATPase	75	0	4	21

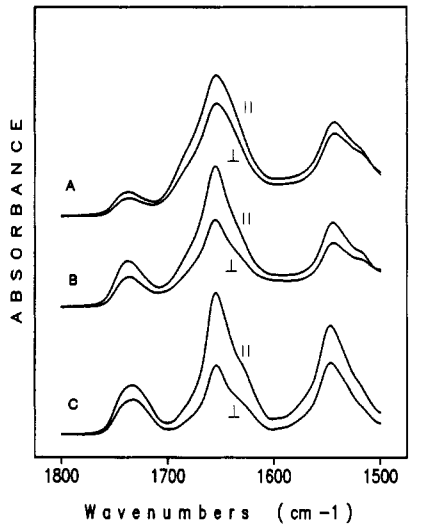

FIGURE 5: Attenuated total reflectance polarized infrared spectra of oriented membranes containing (A) purified (Ca^{2+}+Mg^{2+})-ATPase, (B) T-ATPase, and (C) TP-ATPase. The spectra correspond to the electric vector of the transmitted light polarized parallel (‖) and perpendicular (⊥) to the normal of the film plane.

Analysis of the purified ATPase spectrum yielded 48% α-helix, 20% β-sheet, 14% turns, and 18% random coil (Table 2). However, and for T-ATPase and TP-ATPase, the analysis yielded 62% and 75% α-helix, 9% and 0% β-sheet, 9% and 4% turns and 20% and 21% random coil (Table 2), respectively. According to the PLS method (Dousseau & Pèzolet, 1990; and our own results), these values are reliable to within 7–8%. It is interesting to note that the α-helix content of purified ATPase is similar to already previously published results (Villalaín et al., 1989) (Table 2).

Interestingly enough, the α-helix and random coil content increases as digestion progresses, and at the same time, the content of β-sheet and turns decreases, approaching disappearance (Table 2). According to the most recent models proposed for this protein (Clarke et al., 1989a,b) as detailed above, the intramembranous portion accounts for 203 amino acids whereas the connecting loops located in the luminal side account for 89 amino acids. This means that if our proteolytic digestion has led to a residue formed by these two parts only, the luminal one would include about 30% of the total amino acids. Since we find that 75% of the TP-ATPase corresponds to α-helix, this will agree with the hypothesis that all of the protein stretches inside the membrane have this secondary structure. The interconecting loops outside of the membrane would then account for the random structure. We have done in fact some secondary structure predictions on the major interconnecting loop (W_{855}→E_{895}) using a computer program (Deleage et al., 1987), and the results indicate that this protein stretch is not likely to have α-helical structure.

ATR Polarization Studies. Figure 5 shows the spectra in the 1800–1500 cm^{-1} region of purified ATPase, T-ATPase,

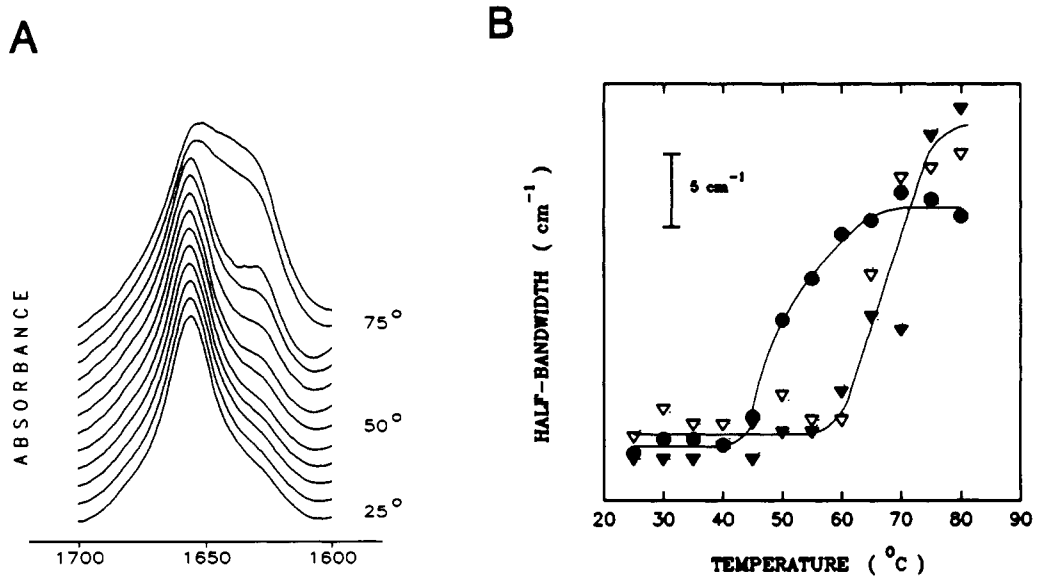

FIGURE 6: (A) Fourier transform infrared spectra of the amide I band of the trypsin plus proteinase K digested (Ca^{2+}+Mg^{2+})-ATPase at different temperatures as indicated. (B) Plot of the half-bandwidth of the amide I band in cm^{-1} versus temperature of (●) (Ca^{2+}+Mg^{2+})-ATPase, (▼) T-ATPase, and (▽) TP-ATPase.

and TP-ATPase, taken with beams perpendicular and parallel polarized to the plane of the membrane film. It can be seen in the spectra of all the samples that the amide I band at 1656 cm^{-1} is more intense in the spectra recorded with the beam polarized parallel to the plane of the lipid film than with the perpendicular one, leading to positive dichroic ratios $R^{ATR} = \Delta A_{\parallel}/\Delta A_{\perp}$, where $\Delta A_{\parallel}$ is the absorption polarized parallel to the plane of incidence and $\Delta A_{\perp}$ is the absorption obtained with a perpendicular polarized beam. In the case of proteins inserted into oriented phospholipid films, R^{ATR} would be always <1 for an α-helix with a random orientation in the plane of the film whereas R^{ATR} would be >1 for an α-helix normal to the plane of the film (Reynaud et al., 1993). The R^{ATR} values obtained in this study were 1.25 for ATPase, 1.61 for T-ATPase, and 2.1 for TP-ATPase. These values can be compared with those obtained previously for other membrane proteins, such as 2.3–2.5 for the Pf1 protein (Azpiazu et al., 1993) and 2.24 for bacteriorhodopsin (Yang et al., 1987), with these membrane proteins considered to have orientations close to the normal to the plane of the bilayer. It is important to remark that upon digestion there is a considerable increase in R^{ATR}, indicating that the orientation of the α-helices was increasingly closer to the perpendicular of the plane of the bilayer.

In order to estimate the mean angle formed by the α-helices and the surface of the membrane, it is necessary to first determine the order parameter of the α-helical part of the protein in the phospholipid bilayer using the expression (Frey & Tamm, 1991; Ishiguro et al., 1993):

$$S = \frac{E_x^2 - R^{ATR}E_y^2 + E_z^2}{[(3\cos^2\alpha - 1)/2](E_x^2 - R^{ATR}E_y^2 - 2E_z^2)} \quad (1)$$

where E_x, E_y and E_z are the electric fields of incident light in the ATR sample [calculated according to Harrick (1967) by using the same coordinate system as in Fringeli and Günthard (1981)], R^{ATR} is the dichroic ratio at 1656 cm^{-1} (see above), and α is the angle between the transition moment of the amide I vibration of an α-helix and the α-helix axis. The calculated order parameter S is considered to be an averaged value over all of the molecules. The order parameter then can be used to obtain the excursion angle, θ:

$$S = \frac{1}{2}(3\langle\cos^2\theta\rangle - 1) \quad (2)$$

which defines the fluctuation of an axially symmetric molecule through the Z axis, i.e., the normal to the surface of the supported bilayer membrane.

Equation 1 may also be used to calculate the order parameter of the phospholipids in the oriented membrane from the intensities of the antisymmetric and symmetric CH_2 stretching vibrations at 2923 and 2854 cm^{-1}, respectively (in this case, α = 90°). It is important to note that the protein order parameters presented above were only evaluated when it was observed that the phospholipids were well-ordered, i.e., when the lipid order parameter was higher than 0.25, indicating that the plane of the membrane is parallel to the ATR crystal (Tamm & Tatulian, 1993).

In order to calculate S, we must know or assume a value for α. For peptide bond amide I modes, α has been estimated variously between 22° and 39° (Tsuboi, 1962; Bradbury et al., 1962; Miyazawa & Blout, 1961; Rothschild & Clark, 1979; Nabedryk & Breton, 1981). Therefore we have measured S as an average value for the assumed α angles in the membrane, in order to know the differences in order parameter between the different protein species used in this work, i.e., purified and digested ATPase. These results indicate that upon digestion there is considerable polarization of the α-helices. It should be kept in mind that these values are the average of all the amide bonds included in α-helices and although they are useful when used for comparison between different samples or proteins a lot of care should be taken before extrapolating from them absolute orientations of the proteins. The few integral membrane proteins whose structure has been studied in detail, such as bacteriorhodopsin (Henderson et al., 1990), show that the α-helices embedded in the membrane are rather oriented and forming relatively small angles with the direction perpendicular to the plane of the bilayer. In summary, our results are compatible with the elimination of the extramembranous portion of the protein, upon digestion, leading to α-helices which are more oriented toward the normal of the bilayer. It is interesting to remember that a recent study of the (Ca^{2+}+Mg^{2+})-ATPase using

cryoelectron microscopy revealed that a substantial part of the membranous portion may have an inclination of ~40° from the vertical (Toyoshima et al., 1993) and therefore although some of the helices may be closer to the normal the expected average values might correspond to an oblique position.

Thermal Denaturation Studies. In order to further study the effect of protease digestion on the enzyme, thermal denaturation studies were performed. The spectra of the TP-ATPase taken at several different temperatures are shown in Figure 6A. It is clearly seen that a broad band, at approximately 1628 cm^{-1}, increases as the temperature is increased, indicating the formation of denatured structures (Susi & Byler, 1986; Byler & Susi, 1986). This band is highly characteristic of thermally denatured proteins; it is believed to represent hydrogen-bonded extended structures possibly of intermolecular nature (Jackson et al., 1991; Muga et al., 1991). This band begins to be observed at 60 °C, whereas at 75 °C there is a significant increase in its intensity, affecting the bandwidth of the whole amide I band. Therefore, the bandwidth at half-height of the amide I band has been used as a denaturation parameter as it is observed in Figure 6B. Whereas the bandwidth of the purified ATPase spectrum has a transition at 45 °C, this transition occurs at about 65 °C for the T-ATPase and the TP-ATPase. Hence, the protein which remains after the digestion is enriched in elements which are more stable against heating than the purified ATPase. This would agree with the intramembranous nature of the residual protein, since it may be assumed that membrane proteins are more resistant to heating denaturation than soluble proteins.

CONCLUDING REMARKS

The results presented in this work clearly indicate that extensive proteolysis of the (Ca^{2+}+Mg^{2+})-ATPase gives rise to a highly hydrophobic residue. Remarkably, the amino acid composition of this residue is very similar to that predicted by previous authors (Bigelow & Inesi, 1992; Clarke et al., 1989a,b) for the segments of this protein located within the membrane plus those located in the lumen. Apart from that, this residue was enriched in α-helical structures (75%), and hence there is a close agreement between this figure and what could be expected according to previously proposed models (Clarke et al., 1989a,b), since according to them and considering only the membranous portion plus the luminal one, about 70% of the amino acids should be in α-helices inside of the membrane. The mean orientation of the α-helical elements was found to be increasingly polarized and closer to the normal to the plane of the membrane, as the (Ca^{2+}+Mg^{2+})-ATPase was increasingly digested. This is in agreement with recent electron diffraction studies (Toyoshima et al., 1993) which show that most of the protein segments located inside of the membrane have this orientation. Denaturation studies show that the residue remaining after proteolysis is enriched in elements more stable to heating than the purified ATPase, in agreement with the notion that hydrophobic proteins embedded within the lipid bilayer should be more stable to heat denaturation than water-soluble proteins. We believe that the approach used in this work should be useful for the study of membrane portions of other membrane proteins, in combination with predictions made from the primary structure and other physical approaches such as electron diffraction.

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