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Characterization of the Secondary Structure and Assembly of the Transmembrane Domains of Trypsinized Na,K-ATPase by Fourier Transform Infrared Spectroscopy*

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Fourier transform infrared spectroscopy has been used to compare native Na,K-ATPase-containing membranes with those trypsinized in the presence of either Rb^+ or Na^+ ions to remove the extramembranous parts of the protein. The protein secondary structure content deduced from the amide I band is approximately 30–35% α-helix, 37–40% β-structure, and 13–15% random coil for native membranes from shark rectal gland and from pig kidney, in both the Na- and K-forms. Trypsinization in either Rb^+ (a K^+ congener) or Na^+ removes approximately 35% of the amide I band intensity of native membranes from shark rectal gland. The protein secondary structural content of the trypsinized membranes lies in the range of approximately 23–32% α-helix, 37–46% β-structure, and 12–18% random coil for the shark and kidney enzymes. The distribution of intensity between the bands corresponding to protonated and deuterium-exchanged α-helices, and between the component bands attributed to β-structure, changes considerably on trypsinization, in the direction of a greater proportion of protonated α-helix and a broader range of frequencies for β-structure. The kinetics of deuteration of the slowly exchanging population of protein amide groups is also changed on trypsinization. The mean rate constant for deuteration of trypsinized membranes is approximately half that for native membranes, whereas the proportion of amides contributing to this population increases on trypsinization. The temperature dependence of the amide I band in the Fourier transform infrared spectra indicates that the onset of thermal denaturation occurs at 58 °C for native membranes (in either Na^+ or K^+) and for membranes trypsinized in Rb^+, but the major denaturation event for membranes trypsinized in Na^+ occurs at approximately 84 °C. These results correlate with the functional properties of the intramembranous section of the enzyme.

The Na,K-ATPase[1] is a large transmembrane protein that is composed of α-subunits (112 kDa) and β-subunits (36 kDa) with equimolar stoichiometry. It transports Na^+ and K^+ across the plasma membrane in an electrogenic antiport fashion coupled to ATP hydrolysis and is responsible for maintaining the ionic balance in the cell. A considerable portion of the enzyme lies outside the membrane. The α-subunit bears the ATP site on the cytoplasmic side of the membrane and is thought to have 10 transmembrane segments (Ref. 1; see also Fig. 1). The β-subunit, on the other hand, is thought to have a single transmembrane segment with the bulk of its extramembranous part, which is highly glycosylated, lying on the extracellular side of the membrane (see Fig. 1).

Considerable progress has been made in simplifying this complex transport enzyme by the finding that extensive trypsinization in the presence of Rb^+ (a K^+ congener) yields a preparation in which a large part of the extramembranous portion of the α-subunit is removed, but the remaining transmembrane fragments retain the ability to occlude Rb^+ ions (2). The tryptic fragments that remain in the membrane have molecular masses in the range 8–12 kDa, consistent with their constituting one or two transmembrane domains; in addition, a larger 19- kDa fragment remains that could constitute a transmembrane four-helix bundle (see Fig. 1). Peptide sequencing of the tryptic fragments indicates that they account for all of the putative transmembrane segments of the α-subunit (3), whereas the β-subunit of the shark enzyme remains intact on trypsinization. For the enzyme from shark rectal gland, trypsinization in the presence of Na^+ leads further to degradation of the 19-kDa membrane-embedded fragment, and concomitantly the Rb^+ occlusion capability is lost (4).

Recently, we have quantitated the lipid-protein interactions and made rotational diffusion measurements with trypsinized preparations of shark Na,K-ATPase membranes (4). From these studies, it was found that trypsinization in RbCl preserves the state of association of the intramembranous domains of the enzyme, whereas on trypsinization in NaCl the intramembranous assembly is disturbed. In the present article, we report complementary studies on the trypsinized enzyme that are performed using Fourier transform infrared (FTIR) spectroscopy. The secondary structure of the native and trypsinized enzyme is analyzed by using the amide I band in the FTIR spectrum. The kinetics of deuteration of the protein are followed to obtain information on the tertiary assembly of the intramembranous domains, and their thermodynamic stability is determined by thermal denaturation experiments.

A very significant result is that the trypsinized Na,K-ATPase preparations still contain a substantial proportion of β-structure, as has been found also for the nicotinic acetylcholine receptor (5) and recently for another P-type ATPase (6).

EXPERIMENTAL PROCEDURES

Na,K-ATPase Preparation and Trypsinization—Membranous Na,K-ATPase was prepared from the rectal gland of *Squalus acanthias* (as described in Ref. 7 but without the treatment with saponin), and from pig kidney outer medulla (as described in Ref. 8) and followed by selective extraction with SDS in the presence of ATP. The specific

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[1] The abbreviations used are: Na,K-ATPase, Na^+/K^+-transporting ATP hydrolase (EC 3.6.1.37); FTIR, Fourier transform infrared; CDTA, *trans*-1,2-cyclohexyldinitrilotetraacetic acid.

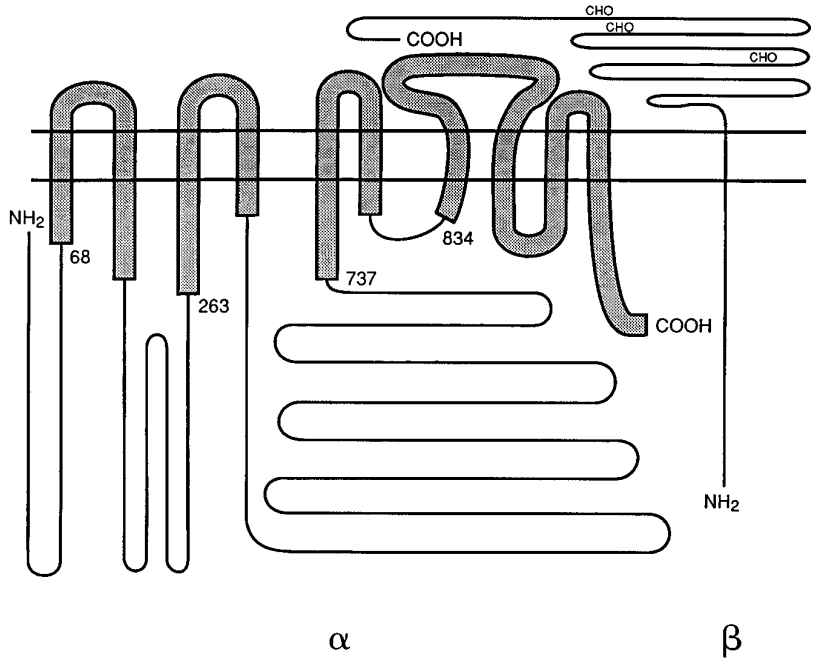

FIG. 1. **Putative transmembrane disposition of the Na,K-ATPase α- and β-subunits (adapted from Ref. 1) with the residual tryptic fragments of the α-subunit indicated by the *hatched areas* (4).** The cleavage positions are also marked, as well as the glycosylation sites on the β-subunit (*CHO*).

activity levels were 1400–1700 and 1000–1600 μmol of ATP hydrolyzed/mg of protein/h at 37 °C for the shark and kidney enzymes, respectively. Reconstitution of solubilized, purified shark Na,K-ATPase was performed as described in Ref. 9.

Na,K-ATPase membranes were trypsinized in the presence of either 10 mM RbCl or 10 mM NaCl, and characterized as described in Ref. 4 for the shark enzyme or in Ref. 2 in the case of the kidney enzyme. Lipid and protein were assayed as described in Ref. 10. Enzyme assays, both ATPase and *para*-nitrophenyl phosphatase, were performed as described in the same reference.

Sample Preparation—Membranes were pelleted at 50,000 rpm for 45 min and subsequently washed with D_2O buffer (30 mM histidine, 1 mM CDTA, pH 7.4, with 30 mM NaCl or KCl), and incubated for 1 day at 4 °C to promote deuteration of the protein amide groups. To study the kinetics of deuteration, membranes were pelleted for 10 min in a benchtop centrifuge (or by ultracentrifugation for the trypsinized membranes) and injected into an FTIR cell at 20 °C. The zero time point was taken as that of the initial resuspension in D_2O buffer.

FTIR Spectroscopy—FTIR spectra were recorded on a Bruker IFS25 spectrometer using CaF_2 cells with 50-μm Teflon spacers in a metal cell housing thermostatted to within 0.1 °C. Except for time-dependent experiments, the spectrometer was flushed with dry nitrogen for 30 min before recording the spectra, to reduce water vapor distortions. Interferograms were accumulated over the spectral range 400–4000 cm^{-1} with a nominal resolution of 2 cm^{-1} and apodized with a triangular function before Fourier transformation. For the kinetic experiments, 10 interferograms, and otherwise 100 interferograms, were recorded.

Band narrowing by Fourier self-deconvolution was performed using a program provided by H. H. Mantsch (National Research Council, Ottawa, Canada) (11). A triangular apodization was used, assuming an initial bandwidth of half-width at half-height (HWHH) = 17 cm^{-1}, and a line-narrowing factor in the range K = 1.5–2.4. The positions of the component bands were determined by fitting Gaussian bands to the spectrum deconvoluted with a value of K = 2.4. The relative intensities of the component bands in the amide I region (1600–1700 cm^{-1}) were determined by band fitting to the non-deconvoluted spectrum (*i.e.* for K = 1). For this band fitting, the positions of the component bands were held fixed at the values determined from the deconvoluted spectrum and the bandwidths, and the relative contribution of Gaussian and Lorentzian components to the bandshapes were allowed to vary in the least-squares minimization. The method used for construction of difference spectra was that described in Ref. 12. Spectra are plotted as absorbance, with the ordinate units given such that the integrated area of the entire band is normalized to 100 × cm^{-1}.

RESULTS

FTIR Spectra and Band Fitting—Representative FTIR spectra in the amide and carbonyl band regions of native shark Na,K-ATPase membranes and those trypsinized in 10 mM Na^+, both suspended in 30 mM Na^+ buffer, are given in Fig. 2. Also shown is the difference spectrum obtained by subtracting the spectrum of trypsinized membranes from that of the native membrane, using the lipid carbonyl band as end point criterion. To remove the carbonyl band, it was necessary to subtract 68% of the integrated intensity from the spectrum for the native membrane, implying that 32% of the protein was removed on trypsinization. Similar results were obtained for native membranes and membranes trypsinized in 10 mM Rb^+, both resuspended in 30 mM K^+ (data not shown). In this case, the subtraction end point corresponded to subtracting 65% of the total intensity from the spectrum of the native membrane. The phospholipid/protein ratios determined for the different preparations were 0.9 ± 0.1, 1.2 ± 0.15, and 1.3 ± 0.15 (w/w) for native membranes and membranes trypsinized in RbCl and NaCl, respectively. The size of the increase in phospholipid/protein ratio on trypsinization is consistent with the loss in the amide band intensity determined by FTIR. Similar FTIR results were also obtained with Na,K-ATPase membranes from pig kidney (data not shown), except that 85% of the intensity was subtracted from native membranes to obtain the difference spectrum end point.

Bands corresponding to carboxylic acid-containing side chains, in the region of 1580–1590 cm^{-1} for aspartic acid and 1560–1580 cm^{-1} for glutamic acid (13), are also seen in Fig. 2. The proportions of these relative to the amide I band intensity that are obtained by band fitting are approximately 11% Asp and 5–6% Glu for native shark membranes (Na-form), and 12% Asp and 7% Glu for trypsinized membranes.

Spectra of the amide I band region that have been subjected to band narrowing by Fourier self-deconvolution are compared for membranes in 30 mM Na^+ and in 30 mM K^+ in Fig. 3. As seen from the difference spectra given in the upper panel, there is very little difference between the Na- and K-forms (E_1 and E_2 conformations, respectively) of the native membranes. For the membranes trypsinized in 10 mM Rb^+ or in 10 mM Na^+ and resuspended in 30 mM K^+ or 30 mM Na^+, respectively, the differences between the Na- and K-forms are somewhat greater than found for the native membranes, but are still relatively small.

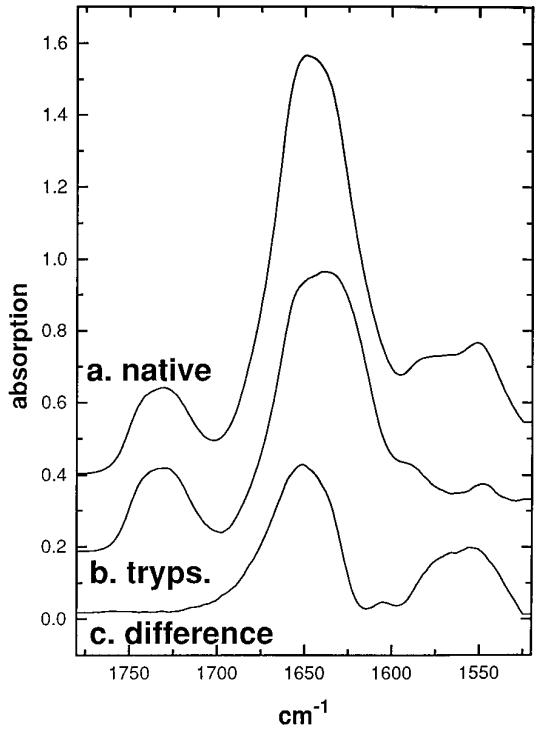

FIG. 2. **FTIR spectra in the amide and carbonyl regions of: native shark Na,K-ATPase membranes (*a*), Na,K-ATPase membranes trypsinized in 10 mM Na^+-containing buffer (*b*), and difference spectrum (*c*) obtained by subtracting 68% of the intensity from *spectrum a* by using *spectrum b*.**

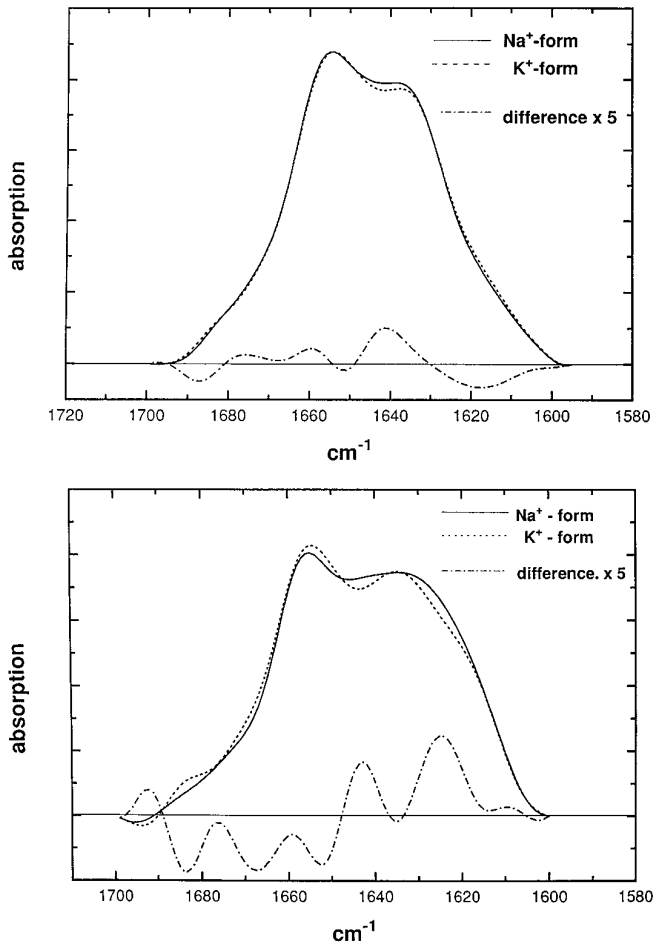

FIG. 3. **Fourier self-deconvoluted FTIR spectra (with band-narrowing factor $K = 1.5$) in the amide I region of shark Na,K-ATPase membranes in the presence of 30 mM K^+ or of 30 mM Na^+.** *Upper panel*, native membranes; *lower panel*, membranes trypsinized in 10 mM Rb^+- or Na^+-containing buffer. The difference spectra between K- and Na-forms are shown by *dashed and dotted lines* in each panel.

Band fitting of the non-deconvoluted spectra, as described under "Experimental Procedures," is illustrated in Fig. 4 for the amide I regions of native and trypsinized membranes. The relative intensities of the component bands are given in Table I. Data are presented for both the Na- and K-forms of the native membranes and for the membranes trypsinized in Na^+ and in Rb^+. Fourier deconvolution, used to determine the component band positions, reveals that the band in the region characteristic of α-helical structures is split, with components at 1649–1654 cm^{-1} and 1657–1661 cm^{-1} corresponding to the positions expected for deuterated and protonated α-helices, respectively (14). A single band is obtained at 1640–1643 cm^{-1} in the region characteristic of random structures in D_2O. Several bands are obtained in the region 1612–1636 cm^{-1} expected for β-sheet and extended structures, along with a weak band at 1674–1678 cm^{-1}. Bands found at 1664–1670 cm^{-1} and 1682–1687 cm^{-1} may possibly be attributable to β-turns. (For assignments see, *e.g.*, Refs. 15 and 16. Possible exceptions to these assignments are discussed in Ref. 17.)

For the native enzyme, there is little difference on the whole in the data on the secondary structure between the Na- and K-forms (Table I and Fig. 2). In addition, differences between the native shark and kidney enzymes are relatively small, except with regard to the distribution of band intensities in the β-region for the Na-form of the shark enzyme. The largest differences are found between the native and trypsinized enzymes, although these differences are mostly in the relative distribution of band intensities within a region assigned to a particular secondary structure. The ratio of protonated to deuterated α-helix is greater for the trypsinized enzymes than for the native enzymes. This is consistent with the amide groups of the membrane-embedded sections being more protected from exchange with D_2O than are those of the extramembranous sections of the protein. The distribution of intensity among the bands assigned to β-structure is also broader in the trypsinized than in the native enzymes. This suggests differences in the precise nature of the β-sheet/extended chain structure between the membrane-embedded and extramembranous parts of the enzyme, although some rearrangement on trypsinization cannot be excluded. Overall, the net secondary structural content is estimated to be approximately 34–35% (30%) α-helix, 38–40% (37–39%) β-structure, 13% (14–15%) random, and 12–13% (16–17%) β-turns for the native shark (kidney) enzymes, and is 23–31% (29–32%) α-helix, 42–46% (37–41%) β-structure, 16–18% (12–13%) random, and 11% (16–18%) β-turns for the trypsinized shark (kidney) enzyme. Results similar to those obtained for the native shark membranes were also obtained for the secondary structural content of shark Na,K-ATPase that had been solubilized, purified, and reconstituted (data not shown).

Amide Deuteration Kinetics—The dependence of the amide I region of the FTIR spectra from Na,K-ATPase membranes on time after transfer from H_2O buffer into D_2O buffer is given in Fig. 5. Progressive changes in the overall band shape are seen corresponding to shifts in the component bands of the peptide amide groups on deuteration (Fig. 5, *upper panel*). The kinetics of amide deuteration can be followed from the difference spectra obtained with increasing time that are given in the *lower panel* of Fig. 5 (see Ref. 12). This method circumvents potential problems in the amide II region of the spectrum that are

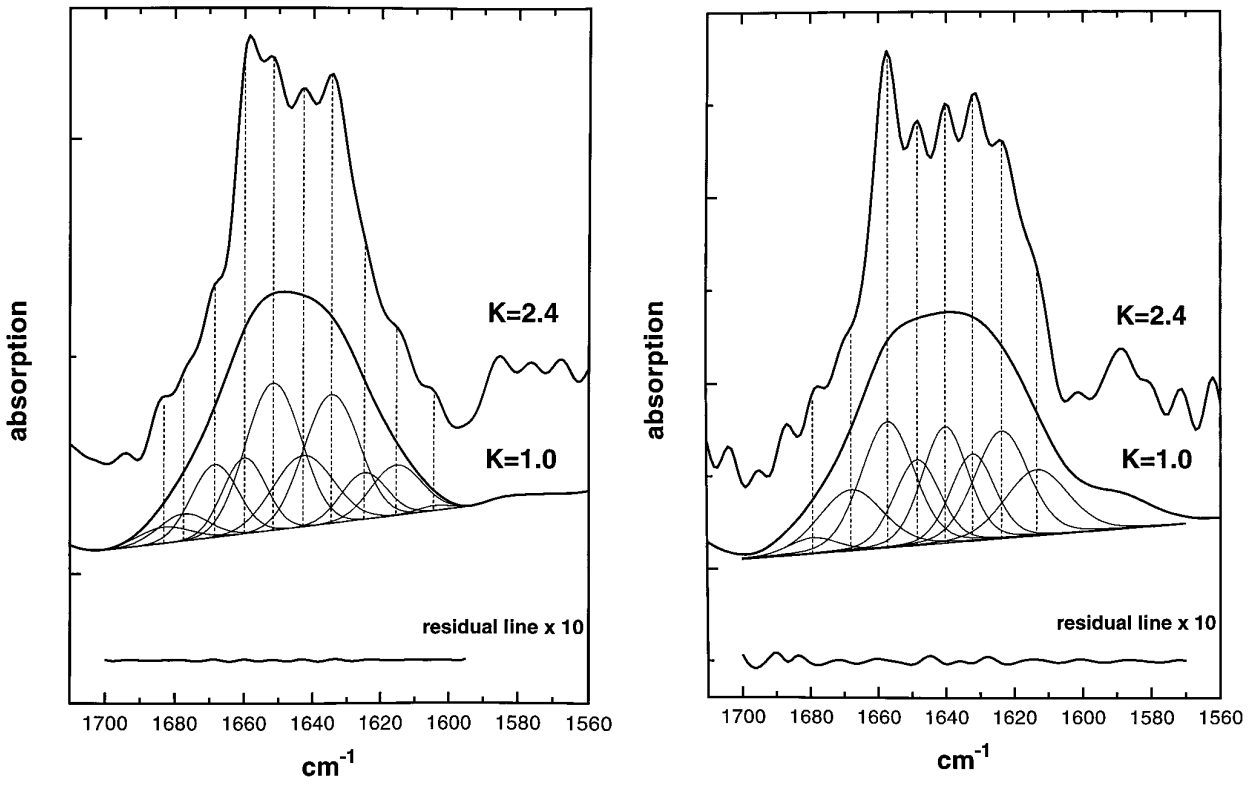

FIG. 4. **Fourier self-deconvoluted FTIR spectra (K = 2.4) and band fitting in the amide I region of the original FTIR spectra (K = 1.0) of shark Na,K-ATPase membranes.** *Left-hand side*, native membranes in the presence of 30 mM Na^+-containing buffer; *right-hand side*, membranes trypsinized in 10 mM Na^+-containing buffer and resuspended in 30 mM Na^+-buffer. Values of the fitted band intensities are given in Table I.

TABLE I

Band positions (cm^{-1}) and relative intensities (%) obtained from fitting the amide I region of the FTIR spectra from native and trypsinized Na,K-ATPase membranes (cf. Fig. 4)

Data are given for membranes from shark rectal gland and from pig kidney.

Assignment	Native Na		Native K		Trypsinized Na		Trypsinized K	
	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%	cm^{-1}	%
Shark								
α-Protonated	1660	10	1660	12	1657	20	1661	12
α-Deuterated	1652	25	1651	22	1649	11	1654	11
Random	1643	13	1642	13	1640	16	1646	18
β-/Extended	1635	22	1634	19	1632	11	1636	14
	1625	6	1623	12	1624	17	1628	13
	1616	8	1614	3	1613	12	1618	17
	1677	4	1678	4	1679	2	1678	2
β-Turn	1668	10	1668	11	1668	11	1670	9
	1683	2	1685	2			1685	2
Other			1607	2			1605	2
Kidney								
α-Protonated	1657	10	1658	10	1658	11	1659	16
α-Deuterated	1649	20	1649	20	1650	18	1649	16
Random	1640	14	1640	15	1640	12	1640	13
β-/Extended	1632	13	1632	13	1631	13	1632	14
	1623	13	1622	14	1621	20	1623	12
	1612	4	1612	3	1613	6	1614	11
	1674	9	1676	7	1676	2		
β-Turn	1664	14	1665	16	1667	13	1669	14
	1686	2	1687	1	1682	5	1685	2
Other	(1604)	(1)	(1605)	(1)			(1602)	(2)

associated with the leakage of background water vapor into the cell at long incubation times. The decrease in intensity of the difference spectra with time (relative to a spectrum obtained at very long times of deuteration) is shown in Fig. 6. This gives the kinetics of deuteration of the slowly exchanging population of amide groups. The proportion of such groups is considerably larger in the trypsinized enzyme than in the native enzyme. Clear differences are seen in the kinetics of amide exchange between native and trypsinized membranes.

The mean rate constants for exchange were obtained from the areas under the deuteration curves in Fig. 6, according to Ref. 12. These were found to have values of $\langle k \rangle$ = 0.0026 and 0.0023 min^{-1} for native membranes in 30 mM K^+ and 30 mM Na^+, respectively, and $\langle k \rangle$ = 0.0013 and 0.0012 min^{-1} for membranes trypsinized in 10 mM Rb^+ and 10 mM Na^+ (and resuspended in 30 mM K^+ and 30 mM Na^+), respectively.

Temperature Dependence of the FTIR Spectra—The dependence of the amide I region of the FTIR spectra from Na,K-

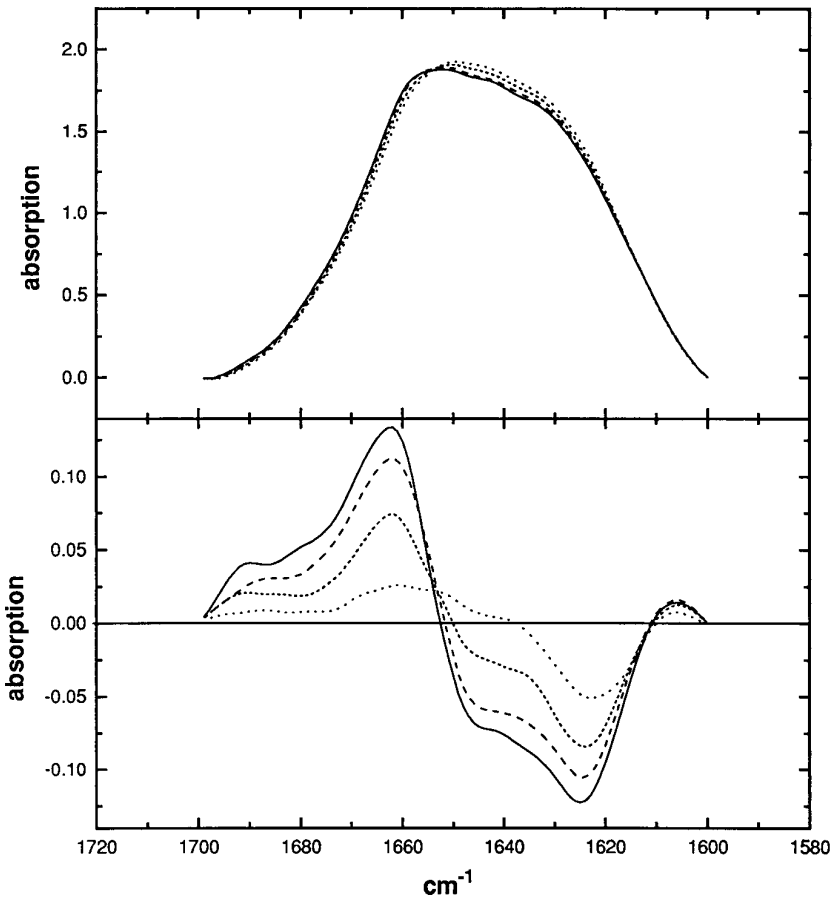

FIG. 5. **Time course of proton-deuteron exchange of the amide groups in shark Na,K-ATPase membranes at 20 °C.** *Upper panel*, FTIR spectra in the amide I region for membranes trypsinized in 10 mM Na^+; from bottom (*solid line*) to top (*dotted line*), the times after suspending in D_2O buffer are 26, 41, 205, and 1605 min, respectively. *Lower panel*, difference spectra obtained by subtracting the spectrum recorded after >4500 min from the spectra in the *upper panel*.

ATPase membranes on temperature is given in Fig. 7. With increasing temperature beyond 57 °C, there is a progressive increase in intensity in the outer regions of the amide I band, at the expense of the central region (Fig. 7, *upper panel*). These changes are seen clearly in the difference spectra constructed by subtracting a normalized spectrum recorded at lower temperature (Fig. 7, *lower panel*). The new bands that appear at higher temperature are those that have been found previously to be characteristic of the thermal denaturation of other proteins (12, 18, 19).

The temperature dependences of the intensities for difference spectra of the type illustrated in Fig. 7 (*lower panel*) are given in Fig. 8 for native membranes in K^+ or in Na^+ and for membranes trypsinized in either Rb^+ or Na^+ and resuspended in K^+ and Na^+, respectively. An abruptly cooperative change in the temperature dependence is seen corresponding to the onset of denaturation in the native membranes (in both Na^+ and K^+) and in the membranes trypsinized in Rb^+, at 58 °C. For membranes trypsinized in Na^+, the temperature dependence is quite different, with the major denaturation event occurring at 84 °C, and only a small change at 58 °C. These results indicate a clear difference in thermal stability of the two trypsinized membranes.

Temperature Dependence of Activity—The time course of product formation on assaying the hydrolytic activity of native shark Na,K-ATPase membranes at various temperatures above that of the standard assay (*i.e.* 37 °C) is shown in Fig. 9. At 45 °C, the initial activity is higher than that at 37 °C, but decreases during the period of the assay, as seen from the non-linearity of the time course for release of inorganic phosphate. At higher temperatures, the activity decreases rapidly with increasing temperature and the enzyme becomes practically inactive at temperatures of 55 °C and above. Similar results were found also for the temperature dependence of the

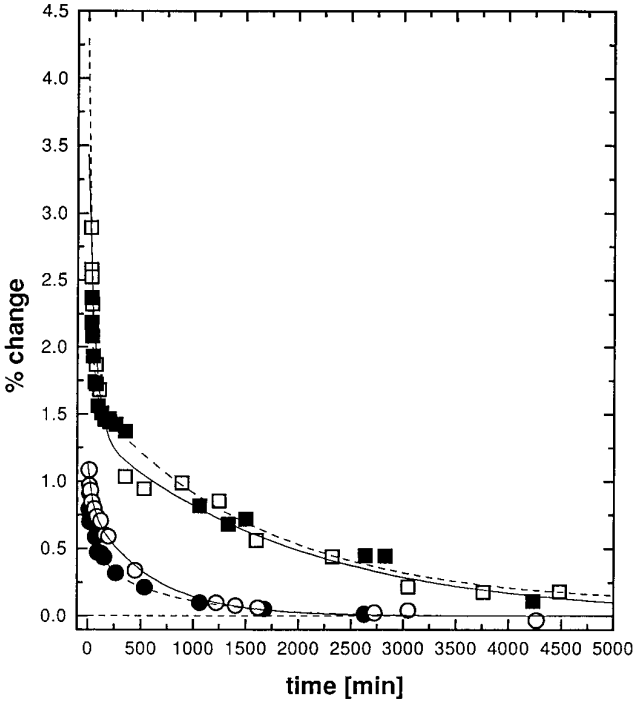

FIG. 6. **Kinetics of deuteration of the amide groups in shark Na,K-ATPase membranes at 20 °C.** The *ordinate* is the area above the baseline in the difference spectra such as those shown in Fig. 5, normalized to the total integrated intensity of the amide I region of the spectrum. *Circles* are data from native membranes, and *squares* are data from trypsinized membranes. *Open symbols* are membranes in 30 mM Na^+ (trypsinized in 10 mM Na^+), and *filled symbols* are membranes in 30 mM K^+ (trypsinized in 10 mM Rb^+).

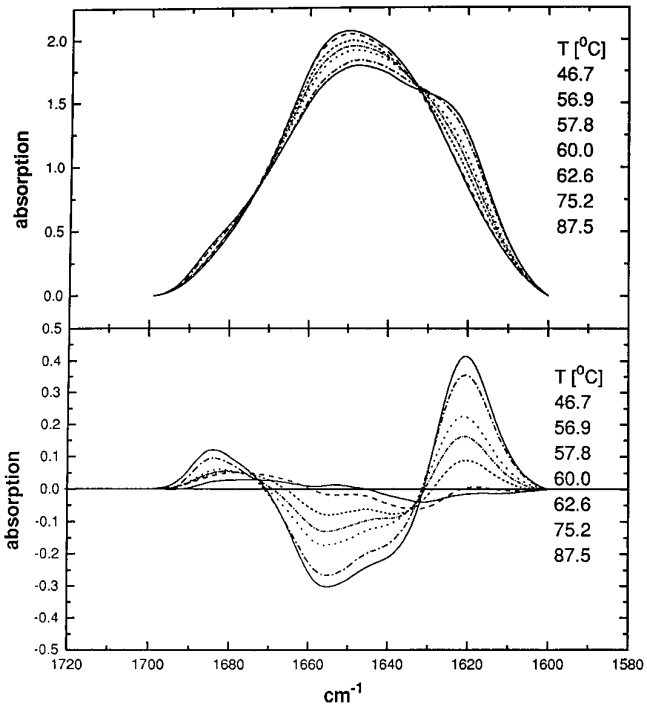

FIG. 7. **Temperature dependence of the amide I region in the FTIR spectra of shark Na,K-ATPase membranes.** *Upper panel*, FTIR spectra of native membranes in 30 mM K^+ recorded at the temperatures indicated (increasing temperature from upper to lower spectrum). *Lower panel*, difference spectra obtained by subtracting the spectrum recorded at 20 °C from those given in the *upper panel*.

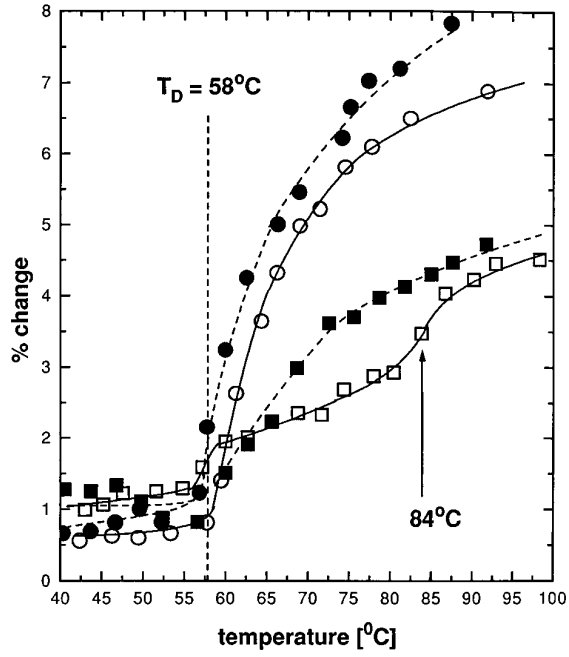

FIG. 8. **Temperature dependence of the area above the baseline in the difference spectra of the type shown in Fig. 7, normalized to the total integrated intensity of the amide I band.** *Circles* are data from native membranes, and *squares* are data from trypsinized membranes. *Open symbols* are membranes in 30 mM Na^+ (trypsinized in 10 mM Na^+), and *filled symbols* are membranes in 30 mM K^+ (trypsinized in 10 mM Rb^+).

potassium-*para*-nitrophenyl phosphatase activity of the native shark enzyme (data not shown).

DISCUSSION

By normalizing the FTIR spectra with respect to the integrated intensity of the lipid carbonyl band, it is found that approximately 35% of the peptide residues of the protein are removed on trypsinizing the shark enzyme (see Fig. 2). Correction for possible differences in the amide I extinction coefficients for different secondary structures will increase this value by less than 20% (*cf.* Ref. 6). This figure for the extent of trypsinization is reasonably consistent with the limited structural data on the protein and the known sites of tryptic cleavage. From image reconstruction studies with electron microscopy, it was estimated that 58% of the protein, including the β-subunit that is not cleaved, is extramembranous (20). From rotational diffusion studies, it was also concluded that 50–70% of the intact protein is external to the membrane (21). The molecular mass of the β-subunit plus the tryptic peptides is 87 kDa (calculated from the amino acid composition), whereas that of the intact $\alpha\beta$-protomer is 147 kDa (*cf.* Fig. 1). Therefore, approximately 40% of the molecular mass is removed on trypsinization, which is in fair agreement with the estimate from FTIR of the proportion of peptide residues that are removed. For the kidney enzyme, the proportion of peptide residues that are removed, as estimated from the amide I band intensities, is considerably smaller. The reason for this is not known. The relative proportions of carboxylic acid side chains identified in the FTIR spectra of the native and trypsinized preparations do not correspond exactly with those estimated from the amino acid composition. The predicted values are 5.4% (4.4%) Asp and 6.6% (6.3%) Glu for the native (trypsinized) enzyme (22, 23). The discrepancy may represent difficulties in band assignment and variable degrees of overlap from residual protonated amide II bands in the FTIR spectra.

Secondary Structure—The results obtained from fitting the amide I band of the intact protein show that it has a relatively high proportion of both α-helical and extended chain (or β-sheet) structure (see Fig. 4 and Table I). Models for the ATP binding site, as well as phosphorylation and transduction domains of P-type ATPases, suggest both α-helical and β-sheet elements for these sections of the protein (24). It is interesting that the part of the protein that is removed on trypsinization is relatively enriched in α-helical structure but nonetheless still contains comparable amounts of both secondary structural elements. Very little difference is seen in the secondary structural content of the Na- and K-forms of the native enzymes, in agreement with previous FTIR studies (25).

Surprisingly, the part of the protein remaining after trypsinization is still relatively enriched in extended chain or β-sheet structures. This β-sheet structure is distinct from that in the parts of the protein that are removed by trypsinization because the distribution of intensity among the component bands in the low frequency β-sheet region of the spectra from the trypsinized preparations differs from that in the FTIR spectra of the whole protein (see Figs. 3 and 4 and Table I). The situation here for the Na,K-ATPase is different from that found for the Ca-ATPase from sarcoplasmic reticulum, where extensive proteolysis resulted in a preparation in which the secondary structure was mostly α-helical (6, 26). However, similar conclusions were drawn recently for a plasma membrane H^+-ATPase from *Neurospora crassa* (6). In this latter work, infrared spectroscopy indicated the presence of β-sheet structures in the membrane-associated domains of this P-type ATPase. These and the present findings are also similar to those of an FTIR study on the proteolytically cleaved nicotinic acetylcholine receptor (5), from which it was concluded that the transmembrane domains were relatively rich in β-structures, in agreement with suggestions based on electron microscope image reconstructions (27). Currently, electron crystallography of

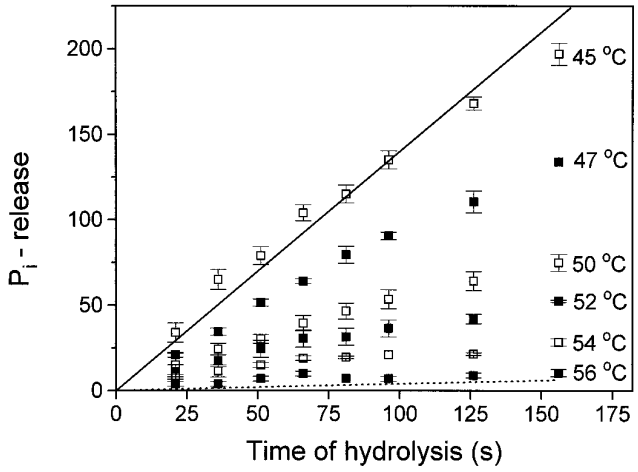

FIG. 9. **Time course of phosphate release (relative units) on ATP hydrolysis by native shark Na,K-ATPase membranes, assayed at various temperatures.** Zero time corresponds to the point at which enzyme was added to the assay medium (130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP, 30 mM histidine, pH 7.4), which was maintained at the temperatures indicated. The *solid line* represents P_i release at 37 °C, corresponding to a specific activity of 1600 μmol of P_i/mg of protein/h. The *dotted line* represents the spontaneous hydrolysis of ATP at 56 °C, in the absence of enzyme.

the Na,K-ATPase does not yet approach this sort of resolution (28).

In the present study, it is not known to what extent the relatively high content of β-type structures of the trypsinized enzyme may be attributable to a rearrangement of the secondary structure in the parts of the tryptic fragments that remain external to the membrane. Although it is possible that the latter may be responsible for the increase of intensity in the flanks of the amide I band, a region of the spectrum that has previously been associated with aggregation processes (29, 30). Nor is it known how much β-structure may be contributed by the β-subunit, which remains intact on trypsinization. However, the extramembranous sections are likely to be relatively small and attempts to shave the preparation more closely have so far proved unsuccessful, although the greater extent of cleavage by trypsinization in NaCl does not lead to an appreciable change in β-structural content relative to trypsinization in RbCl. In addition, simple calculations show that, even if it were entirely in a β-sheet/extended chain conformation (which is highly unlikely), the intact β-subunit is hardly sufficient to account for the whole of the β-structural content of the trypsinized enzyme. Therefore, it seems most likely that part, possibly an appreciable part, of the intramembranous domain of the α-subunit of the Na,K-ATPase has a β-sheet or extended chain structure.

Amide Exchange—The average rate of proton-deuteron exchange for the populations of amide groups that exchange slowly is faster in the native enzyme than in the trypsinized preparation. This is consistent with a greater proportion of these amide groups being buried within the membrane in the trypsinized enzyme. Correspondingly, the proportion of residual protonated α-helical structures is also found to be greater in the trypsinized enzyme (cf. Fig. 4). The amide exchange results provide no evidence for the existence of a pore-like structure through which the ions could pass and which is unmasked to give rapid amide exchange on removing extramembranous parts of the protein. This negative result is consistent with the fact that membranes trypsinized in RbCl are still able to occlude Rb^+ ions.

Also in correspondence with the relative proportions of protonated and deuterated α-helical structures found in the static experiments of Fig. 4, the proportion of slowly exchanging amide groups in the kinetic experiments is larger in the trypsinized membranes than in the native membranes (see Fig. 6). This is true even if the kinetic data for the trypsinized preparations are corrected down by a factor of 0.65–0.68 to represent their contribution to the total amide population in the native enzyme. It is possible that this excess of slowly exchanging amide groups represents those that are not exchanged at all in the native enzyme (as evidenced by the larger residual protonated amide II band in the difference spectrum in Fig. 2 (*trace c*)) and become partially accessible to water after the proteolytic cleavage.

Thermal Denaturation—Results on structural aspects of the thermal denaturation of the Na,K-ATPase have, to the best of our knowledge, not been reported previously. Denaturation of the native enzyme occurs at approximately 58 °C, a temperature considerably higher than that at which appreciable loss of activity occurs on prolonged incubation (31). Under the conditions used here, this denaturation process is irreversible. It is accompanied by the appearance of bands in the FTIR spectrum that have previously been attributed to the formation of intermolecular hydrogen bonds, resulting from an irreversible aggregation of the protein (29, 30). The temperature found here for the onset of denaturation of the Na,K-ATPase is considerably higher than that reported (45 °C) from FTIR studies of the Ca-ATPase (26). The thermal denaturation is mirrored by the temperature dependence of the activity of the native shark enzyme in membranous preparations. Both the Na,K-ATPase and potassium-*para*-nitrophenyl phosphatase activities are high at 45 °C, but decrease rapidly at higher temperatures, tending toward zero immediately below the denaturation temperature.

The denaturation temperature obtained by FTIR for the enzyme trypsinized in RbCl is identical to that found for the native enzyme. This is an interesting result, because it implies that the thermodynamic stability of the enzyme structure (although not necessarily its activity) is controlled by the intramembranous domains of the protein. The major denaturation event for the enzyme trypsinized in NaCl is shifted to considerably higher temperature, implying a significant rearrangement in the intramembranous domains of the protein. Recently, it has been shown that thermal unfolding of the Na,K-ATPase α-subunit exposes to the extracellular side epitopes, which, in the native protein, are within the transmembrane segments, and that simultaneously the C terminus becomes exposed to the extracellular side, whereas it is located on the intracellular side in the native conformation (32). These parts of the protein are contained within the 19-kDa fragment. It is therefore tempting to speculate that the denaturation patterns observed for native as well as for Rb-trypsinized enzymes are similar because structural intactness of the C terminus of the α-chain is maintained, and that the difference between thermal denaturation in Rb- and Na-trypsinized membranes arises because the 19-kDa fragment is not preserved when the α-subunit is trypsinized in the presence of NaCl. This is also in agreement with spin label studies, which found that, whereas the rotational mobility of the intramembranous section of the protein was unchanged on trypsinization in RbCl, it was decreased appreciably on trypsinization in NaCl (4). In addition, the Rb occlusion function that is retained by membranes trypsinized in RbCl was lost on trypsinization in NaCl. A similar study on the Ca-ATPase reported a significant increase in denaturation temperature on proteolysis (26), to a level comparable to that found here for the native Na,K-ATPase. Whether this implies that the intramembranous do-

mains of the Ca-ATPase have retained functional assembly on proteolysis is not clear.

Conclusions—The present FTIR results on proteolytically shaved Na,K-ATPase membranes suggest that the intramembranous domains may contain appreciable β-secondary structure. At least part of the amide groups in these intramembranous structures are less accessible for deuterium exchange than are those in the extramembranous domains. The functional assembly of the intramembranous domains of the enzyme trypsinized in RbCl extends to a thermal stability that is characteristic of the native enzyme.

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