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Fourier transform infrared spectroscopic studies on gastric H^+/K^+-ATPase

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Suspensions of membrane-bound H^+/K^+-ATPase in both H_2O and ^2H_2O were investigated using Fourier transform infrared (FT-IR) spectroscopy. Second-derivative techniques were used to reveal the overlapping bands in the 1800–1500 cm^{-1} region. Analysis of the amide I band shows that the protein component contains substantial amounts of both α-helical and β-sheet structures. Addition of 10 mM KCl to a suspension in ^2H_2O does not significantly affect the amide I band, indicating that the E_1–E_2 conformational transition of the enzyme, induced by K^+, does not involve a gross change in protein secondary structure. Analysis of the amide II band in the spectra of suspensions in ^2H_2O shows that inhibition of the enzyme with omeprazole increases the rate of ^1H–^2H exchange, indicating an increase in conformational flexibility. Furthermore, an additional feature at 1628 cm^{-1} in the spectra of the inhibited samples in ^2H_2O could either support a conformational change or arise from a vibrational mode of omeprazole in its enzyme-bound form. The frequency of the band due to the symmetric stretching vibrations of the methylene groups of the lipid acyl chains increases steadily with increasing temperature indicating that there is no co-operative melting process in the lipid component of the membrane over the temperature range 9–50 °C. For comparison, FT-IR studies on aqueous suspensions of Na^+/K^+-ATPase were also carried out. These show that the protein components in the Na^+/K^+- and H^+/K^+-ATPases have similar secondary structures.

Introduction

The secretion of acid by the parietal cell is mediated by gastric H^+/K^+-ATPase [1–3]. Clearly, knowledge of the structure of the enzyme is important for an understanding of its function. The primary structure of rat stomach H^+/K^+-ATPase has recently been deduced from its cDNA clone [4]. However, very little is known about the secondary structure of the enzyme (some of the preliminary results from the present study have been reported [5]). Using Fourier transform infrared (FT-IR) spectroscopy, we, therefore, investigated the secondary structure of the protein component in some membrane-bound H^+/K^+-ATPase samples and, for comparison, we also investigated the structure of the protein component in a Na^+/K^+-ATPase sample. Previous infrared studies on this latter enzyme have shown that it contains roughly equal proportions of ordered (α-helical, β-sheet) and disordered structures and that there is little or no difference in the

Abbreviations: FT-IR spectroscopy, Fourier transform infrared spectroscopy; Pipes, 1,4-piperazinediethanesulphonic acid.

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secondary structures of the Na^+ and K^+ forms [6–8]. H^+/K^+-ATPase can also exist in two forms; either an E_1 or an E_2 conformation [9]. We, therefore, explored whether these two conformations can be distinguished by FT-IR spectroscopy.

Omeprazole is a potent inhibitor of gastric acid secretion both in vitro [10] and in vivo [11]. It acts by binding irreversibly to thiol groups on H^+/K^+-ATPase [12]. In order to determine whether this induces any change in the secondary structure of the protein, we compared FT-IR spectra of inhibited and uninhibited enzyme samples.

Activities of membrane-bound enzymes are frequently determined at several temperatures. Under these circumstances, it is important to establish whether the lipid component of the membrane undergoes a phase transition over the temperature range of interest. Accordingly, we used FT-IR methods to study the effect of temperature on the lipid component of membrane-bound H^+/K^+-ATPase.

The technique used for these studies, FT-IR spectroscopy, is now being widely applied to biological systems [13,14]. It offers the advantage of being able to investigate the lipid and protein components of a biological membrane simultaneously, without the need for probe molecules. In particular, the band, at approx. 2850 cm^{-1}, due to the symmetric stretching vibrations of the methylene groups in the lipid acyl chains is sensitive to the degree of order of the bilayer, whilst the overlapping components of the amide I band, at approx. 1650 cm^{-1}, provide information about the secondary structural composition of the protein. These overlapping components can be analysed by the use of derivative [15], Fourier self-deconvolution [16] and band-fitting techniques [17]; we used the former for the studies described in this paper. Additionally, we examined samples in both H_2O and ^2H_2O, because recent studies have shown that it is advisable to record spectra of proteins in both these solvents in order to enable reliable assignments of the amide I bands [18,19].

Materials and Methods

Gastric vesicles of H^+/K^+-ATPase were prepared from pig fundic mucosa as described previously [20]. Essentially, tissue was homogenised in isolation medium (0.25 M sucrose containing 5 mM Pipes/Tris buffer, pH 7.4) using a Teflon-glass homogeniser. A microsomal fraction was obtained which was further separated by discontinuous density ultracentrifugation. That material present at the interface between isolation medium and isolation medium containing 9% (w/v) Ficoll contained intact gastric vesicles. To obtain lyophilised vesicles, the same interface material was centrifuged in hypotonic solution and the resulting pellet was resuspended in 5 mM Tris-Cl buffer (pH 7.4) and freeze-dried. The sample of Na^+/K^+-ATPase, isolated and purified from duck salt gland by a procedure [21] based on the method of Jorgensen [22], was a gift from Dr. A.A. Boldyrev, Department of Biochemistry, Moscow State University, Moscow, U.S.S.R. It was washed and resuspended in 5 mM Tris-Cl (pH 7.4) and an aliquot was freeze-dried. All the samples were stored at $-70\,^\circ C$ prior to spectroscopic examination.

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [23]. Analysis of intact and lyophilised gastric vesicles of H^+/K^+-ATPase indicated the presence of a 100 kDa polypeptide accounting for between 50 and 60% of the total Coomassie blue staining material. Analysis of the sample of Na^+/K^+-ATPase again indicated a 100-kDa polypeptide accounting for more than 90% of total stain.

Omeprazole-bound gastric vesicles were prepared by incubating lyophilised vesicles (100 μg protein/ml) for 30 min at $37\,^\circ C$ in 10 mM Pipes/Tris buffer (pH 6.1) in the presence of 100 μM [*benzimidazole*-2-^{14}C]omeprazole. The incorporation of radiolabel into this material was determined by filter binding as described previously [20] and corresponded to a level of 23.2 ± 2.8 nmol omeprazole per mg protein (range, $n = 2$ experiments). This level of incorporation resulted in a complete inhibition of K^+-ATPase activity. Membrane-bound material was recovered by centrifugation at $100\,000 \times g$ for 60 min. The pellet was resuspended in 5 mM Tris-Cl buffer (pH 7.4) and was freeze-dried. [*benzimidazole*-2-^{14}C]Omeprazole was synthesised at Smith Kline & French [24]. All other materials were of reagent grade. Protein concentrations were measured by the method of Lowry et al. [25].

For the FT-IR studies, freeze-dried samples, already containing buffer salts, were resuspended at room temperature (approx. 20 °C) in either H_2O or ^2H_2O (99.8 atom% ^2H, obtained from Sigma). The time of addition of ^2H_2O was noted in order to enable the monitoring of the ^1H-^2H-exchange process. To investigate the effect of K^+ on H^+/K^+-ATPase, an aliquot (less than 5% of the volume of the suspension) of a concentrated KCl solution in ^2H_2O was added to a suspension of H^+/K^+-ATPase in ^2H_2O.

Samples were placed in a Specac 20500 heatable cell fitted with CaF_2 windows and a spacer; a 6-μm tin spacer was used for the suspensions in H_2O and a 50-μm Teflon spacer was used for the suspensions in ^2H_2O. The cell was held in a water-jacketted holder supplied with water from a Neslab EX100DD circulating bath, the temperature of which could be changed at 0.1 C° intervals over the range 5–80 °C. The stability of the temperature at the cell window, measured via a thermocouple, was better than ±0.1 C°. Each sample was allowed to equilibrate in the cell for at least 15 min, at a chosen temperature, before its spectrum was recorded using a Perkin-Elmer (P-E) Fourier transform infrared spectrometer (P-E 1750) equipped with an interleave accessory and a TGS detector. 64 and 400 scans were averaged for the ^2H_2O and H_2O suspensions, respectively, at a resolution of 4 cm^{-1} using a medium Norton-Beer function for apodization. The sample compartment was continuously purged with dry nitrogen during data acquisition.

Difference spectra were generated using the P-E interactive difference routine (IDIFF) to subtract the spectrum of the buffer from the spectrum of the suspension; both spectra having been recorded under identical conditions in the same cell. A factor was chosen which gave a flat baseline from 1900 to 1750 cm^{-1}. The difference spectra of samples in ^2H_2O contained bands at 3402 and 1461 cm^{-1} due to ^2HOH. These were removed by the interactive subtraction of a difference spectrum of ^2HOH (1% (v/v) of H_2O in ^2H_2O) using a factor which gave a flat baseline from 4000 to 3400 cm^{-1}. Second-derivative spectra were generated from the difference spectra using the P-E derivative routine (DERIV) with a width of 13 points. Weak sharp bands due to water vapour were occasionally observed in the second-derivative spectra between 1800 and 1500 cm^{-1}. These were removed by the interactive subtraction of a second-derivative spectrum of water vapour using a factor which gave the smoothest baseline between 1800 and 1775 cm^{-1}. The peak position of the band at approx. 2850 cm^{-1}, arising from the symmetric stretching vibrations of the methylene groups in the lipid acyl chains, was determined from the second-derivative spectrum to the nearest 0.05 cm^{-1}.

Results

The difference spectrum of intact vesicles of H^+/K^+-ATPase in H_2O is shown in Fig. 1a. The broad complex bands at approx. 1654 and approx. 1550 cm^{-1} are the amide I and amide II bands of

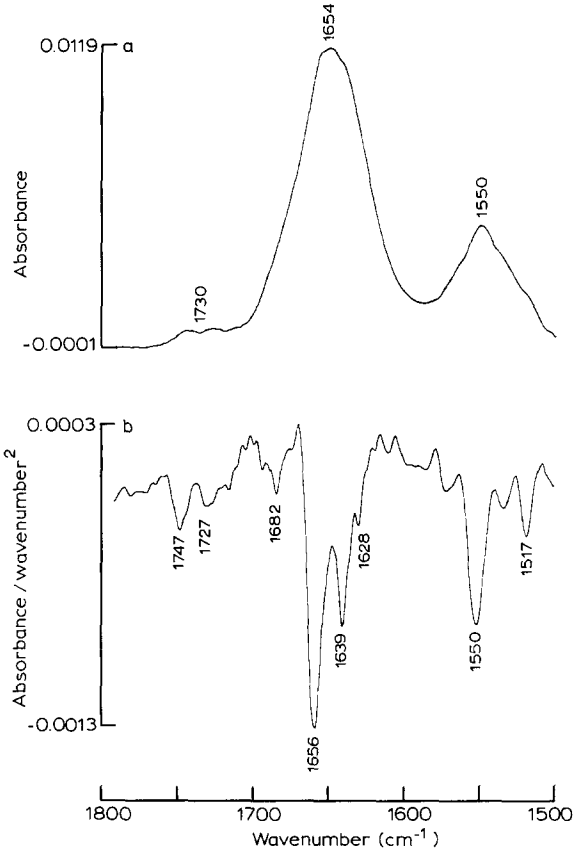

Fig. 1. Difference (a) and second-derivative (b) spectra of intact vesicles of H^+/K^+-ATPase in sucrose/H_2O buffer (pH 7.4), 2% (w/v), 20 °C.

the peptide linkages, respectively [26], whilst the weak bands at approx. 1730 cm^{-1} can be assigned to the stretching vibrations of the ester carbonyl groups of the lipid acyl chains [26]. The components of these broad features are revealed in the second-derivative spectrum (Fig. 1b). Based on previously published work, the following assignments can be made: the weak band at 1682 cm^{-1} to antiparallel β-sheet structures and/or β-turns [27,18,19]; the strong band at 1656 cm^{-1} to α-helical and/or disordered structures [27]; the band at 1639 cm^{-1} to antiparallel β-sheet structures [27]; the band at 1550 cm^{-1} to amide II; the band at 1517 cm^{-1} to tyrosine residues [28]; the bands at 1747 and 1727 cm^{-1} to carbonyl groups of the *sn*-1 and *sn*-2 lipid acyl chains, respectively [29]. The assignment of the weak band at 1628 cm^{-1} is uncertain; it could be due either to another type of β structure or to side-chain residues.

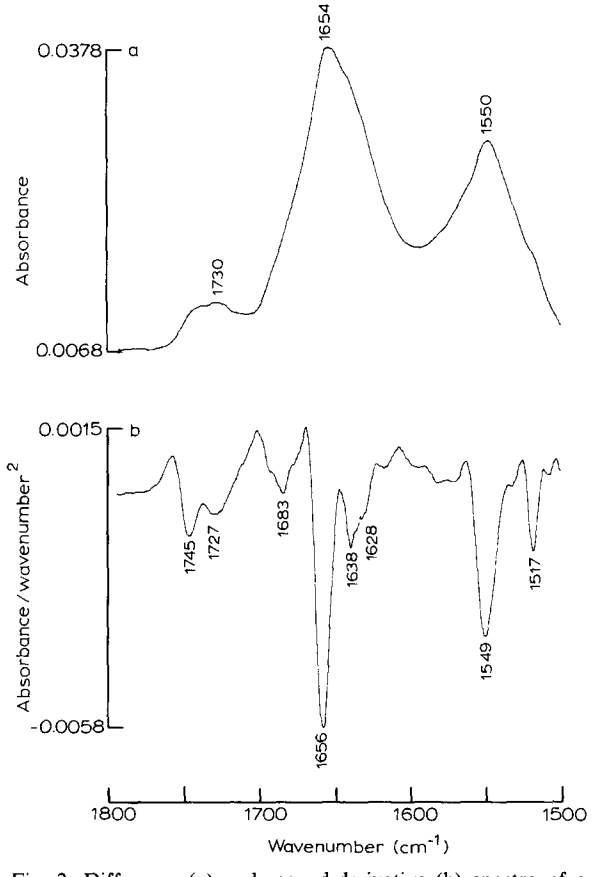

Fig. 2. Difference (a) and second-derivative (b) spectra of a freeze-dried samples of H^+/K^+-ATPase in H_2O (pH 7.4), 2.5% (w/v), 25 °C.

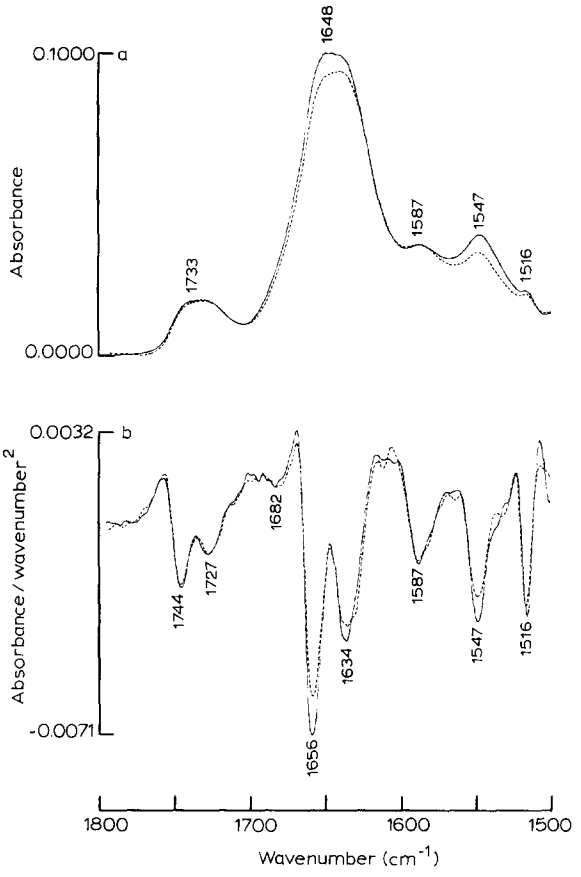

Fig. 3. Difference (a) and second-derivative (b) spectra of a freeze-dried sample of H^+/K^+-ATPase in ^2H_2O (p^2H 7.4), 1% (w/v), 25 °C. The spectra were recorded 22 min (solid line) and 366 min (broken line) after the addition of ^2H_2O.

Fig. 2a and b show the difference and second-derivative spectra, of a freeze-dried sample of H^+/K^+-ATPase suspended in H_2O. The spectra are very similar to the corresponding spectra of the intact vesicles (Fig. 1). The apparent reduction in the intensity of the β-sheet band at 1639 cm^{-1} could be due to the uncertainties encountered when subtracting away the strong water band at 1646 cm^{-1} to obtain the difference spectra. It is unlikely to be indicative of a significant change in protein secondary structure.

Fig. 3a presents the difference spectra of a freeze-dried sample of H^+/K^+-ATPase, 22 and 366 min after the addition of ^2H_2O. Comparison with Fig. 2a shows that there is a large reduction

in the intensity of the amide II band after 22 min – due to $^1H-^2H$ exchange – followed by a further smaller reduction after 366 min. A spectrum recorded after 50 h (not shown) is virtually identical to that recorded after 366 min, indicating that $^1H-^2H$ exchange reaches equilibrium within 366 min. Comparison of Figs. 2a and 3a also shows that $^1H-^2H$ exchange results in a shift of the amide I band to lower frequencies.

The second derivatives of the spectra in Fig. 3a are given in Fig. 3b. The residual amide II band at 1547 cm^{-1} is clearly resolved from both the tyrosine band at 1516 cm^{-1} and a higher frequency band at 1587 cm^{-1}. This latter band is absent or very weak in Fig. 2b which suggests that it is associated with $^1H-^2H$ exchange; however, its exact origin has still to be established. The band at 1656 cm^{-1}, which has not shifted from its position in Fig. 2b, can be unambiguously assigned to α-helical structures because disordered structures in ^2H_2O are known to absorb at approx. 1644 cm^{-1} [27]. The absence of a shift in the band at 1656 cm^{-1} indicates that the α-helical structures are relatively inaccessible to ^2H_2O. By contrast, the β-sheet structures must be accessible to ^2H_2O because the band at 1634 cm^{-1}, assigned to such structures, has shifted to lower frequencies by 5 cm^{-1} from its position in Figs. 1b and 2b.

The second derivative (not shown) of the amide I band of the Na^+/K^+-ATPase sample in ^2H_2O also shows only two features at 1656 and 1634 cm^{-1}. This indicates that the protein component of the Na^+/K^+-ATPase sample has a very similar secondary structure to that of the H^+/K^+-ATPase sample.

Fig. 4 compares the second-derivative spectra of a freeze-dried sample of H^+/K^+-ATPase in ^2H_2O, with and without 10 mM KCl. The KCl has no effect on the bands at 1656 and 1634 cm^{-1} suggesting that it has no significant effect on the secondary structure of the protein; in addition, it has no effect on the intensity of the residual amide II band at 1547 cm^{-1} relative to the tyrosine band at 1516 cm^{-1}, indicating that it does not alter the extent of $^1H-^2H$ exchange at equilibrium. The second-derivative spectrum of the sample in H_2O (Fig. 2b) is also virtually identical to the second-derivative spectrum (not shown) of the same sample after the addition of 10 mM KCl.

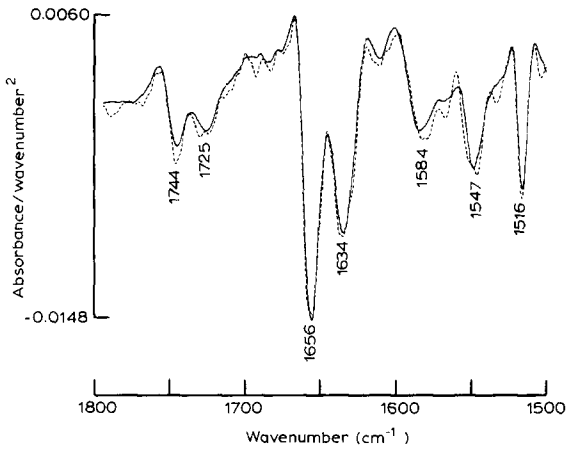

Fig. 4. Second-derivative spectra of a freeze-dried sample of H^+/K^+-ATPase in ^2H_2O (p^2H 7.4), 1% (w/v), 25 °C. Solid line, no KCl; broken line in the presence of 10 mM KCl.

Fig. 5 presents the second-derivative spectra of a freeze-dried sample of omeprazole-inhibited H^+/K^+-ATPase, 21 and 365 min after the addition of ^2H_2O. These spectra should be compared with those of the uninhibited sample (Fig. 3b). Based on the intensity of the residual amide II band at 1547 cm^{-1} relative to the tyrosine band at 1516 cm^{-1}, the extent of $^1H-^2H$ exchange in the inhibited sample compared to the uninhibited sample is greater after both 21 and 365 min. In fact, the extent of exchange of the inhibited sam-

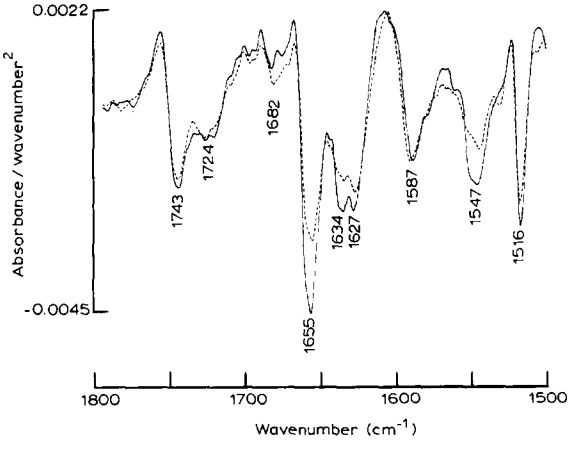

Fig. 5. Second derivative spectra of a freeze-dried sample of omeprazole-inhibited H^+/K^+-ATPase in ^2H_2O (p^2H 7.4), 1% (w/v), 25 °C. The spectra were recorded 21 min (solid line) and 365 min (broken line) after the addition of ^2H_2O.

ple after only 21 min is similar to that of the uninhibited sample after 366 min. Another difference is the appearance of the additional feature at 1627 cm^{-1} in the spectrum of the inhibited sample. The above differences were observed in three separate experiments and could be indicative of drug-induced conformational changes in the enzyme.

The difference spectrum, in the NH and CH stretching regions, of a H^+/K^+-ATPase sample, 366 min after the addition of ^2H_2O, is shown in Fig. 6. The weak amide A band at 3280 cm^{-1} confirms the presence, at equilibrium, of some unexchanged peptide NH groups. The relative sharpness of the band (half-bandwidth approx. 85 cm^{-1}) suggests that the peptide groups are contained in regular structures [30]. (Note that this amide A band is only revealed after the interactive subtraction of a band at 3402 cm^{-1} assigned to the stretching vibration of the OH group in ^2HOH.) The bands between 3000 and 2800 cm^{-1} arise mainly from the lipid acyl chains. The asymmetric and symmetric CH_3 stretching bands appear at 2953 and 2874 cm^{-1}, respectively, whilst the antisymmetric and symmetric CH_2 stretching bands are observed at 2923 and 2852 cm^{-1}, respectively. This latter band is free from interference from underlying protein features and its temperature dependence can be used to obtain information about the order and mobility of the membrane [31]. Fig. 7 shows that the frequency

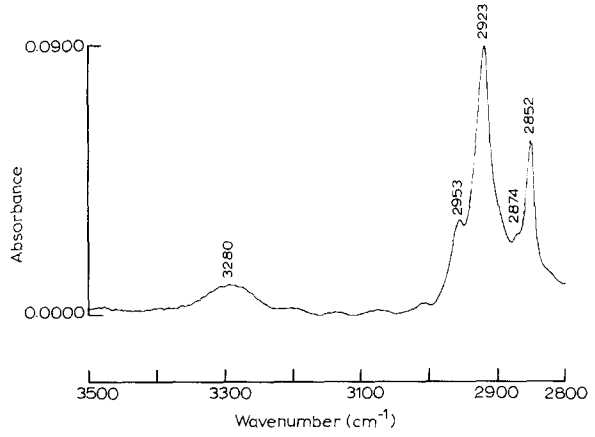

Fig. 6. Difference spectrum of a freeze-dried sample of H^+/K^+-ATPase in ^2H_2O (p^2H 7.4), 1% (w/v), 25 °C. The spectrum was recorded 366 min after the addition of ^2H_2O.

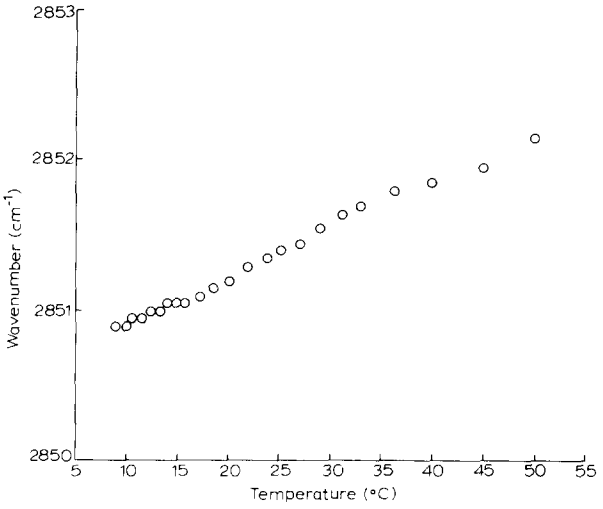

Fig. 7. Plot of temperature vs. wavenumber for the band at 2852 cm^{-1} in Fig. 6.

maximum of the band increases steadily with increasing temperature without any abrupt shift indicative of a phase change. An almost identical plot (not shown) is obtained for the intact vesicles.

Discussion

The second-derivative spectra of the H^+/K^+-ATPase samples reported in Figs. 1–3 show that the protein components of the samples contain substantial amounts of both α-helical and β-sheet structures. There is no clear evidence for the existence of disordered structures. The same conclusion can be reached for the Na^+/K^+-ATPase sample. Brazhnikov et al. [6] have reported that Na^+/K^+-ATPase in ^2H_2O contains approx. 55% of disordered structures. These structures would be expected to give rise to a band at approx. 1644 cm^{-1} in the second-derivative spectrum [27]. The absence of a band at this position suggests that either the percentage of disordered structures in the Na^+/K^+-ATPase sample is considerably less than 55% or that the band due to disordered structures is much broader than the bands due to α-helical and β-sheet structures and, consequently, gives rise to a relatively weak feature in the second-derivative spectrum. Certainly, considerable caution must be exercised when drawing quantitative conclusions from second-derivative spectra,

because the intensity of a second-derivative band is inversely related to the square of the width of the band in the original zero-order spectrum [32–34]. However, the sensitivity of the second-derivative spectrum to changes in peak frequency and bandwidth can be turned to advantage when investigating conformational changes induced by ligands.

The effects of two ligands, K^+ and omeprazole, on H^+/K^+-ATPase have been investigated in this paper. K^+ has been reported to induce an E_1–E_2 conformational transition in the enzyme [9]. However, addition of 10 mM KCl to a H^+/K^+-ATPase sample in ^2H_2O has no effect on the amide I bands in the second-derivative spectrum (Fig. 4). This indicates that the E_1–E_2 conformational transition does not involve a gross change in protein secondary structure, but presumably only a local change which is not readily detectable by FT-IR spectroscopy. Infrared [8] and circular dichroism [35] studies on Na^+/K^+-ATPase have also shown that the secondary structure of this closely related enzyme does not change when it is cycled between the two major conformational states, E_1 and E_2.

Comparison of Figs. 3b and 5 shows that the other ligand investigated in this paper, omeprazole, does induce some changes in the H^+/K^+-ATPase sample in ^2H_2O. It increases the extent of ^1H–^2H exchange at equilibrium – monitored by the intensity of the amide II band at 1550 cm^{-1} relative to the tyrosine band at 1516 cm^{-1} – suggesting that it increases conformational flexibility, thus making some of the peptide groups more accessible to the ^2H_2O. These groups are more likely to be in the β-sheet regions, because it is the band assigned to the β-sheet structures (at 1639 cm^{-1} in Fig. 2b) rather than the band assigned to α-helical structures (at 1656 cm^{-1} in Fig. 2b) which shifts on ^1H–^2H exchange.

Omeprazole is thought to exert its inhibitory action by covalent acid-activated reaction with essential thiol groups on the enzyme [36]. The number of covalently incorporated omeprazole molecules required for complete inhibition can vary between two and thirteen per catalytic phos phorylation site, depending upon the means by which this inhibitor is acid-activated [37]. Furthermore, additional incorporation of omeprazole can occur beyond the point of complete inhibition [20]. The method used in this study was intended to maximise the number of omeprazole molecules incorporated into the enzyme, each of which introduces an additional charged species [38]. It is not unreasonable that this leads to an increase in conformational flexibility. A conformational change is possibly supported by the appearance of an additional feature at 1627 cm^{-1}, in the spectrum of the inhibited sample (Fig. 5). Its position suggests that it may be due to a perturbed β-sheet structure. However, the possibility that the band arises from a vibrational mode of omeprazole in its bound form cannot be discounted. We have yet to assess whether similar changes occur to the enzyme following inhibition by omeprazole under conditions that minimise incorporation.

The lipid component of membrane bound H^+/K^+-ATPase does not undergo a phase transition over the temperature range 9–50 °C (Fig. 7); the frequency of the symmetric stretching vibrations of the methylene groups in the lipid acyl chains increases steadily from 2850.9 cm^{-1} at 9 °C to 2852.15 cm^{-1} at 50.1 °C, indicative only of a gradual increase in chain disorder. Similar results have been reported for native sarcoplasmic reticulum [39]. A recent study of the lipid composition of vesicles prepared from pig gastric mucosa [40], which is in reasonable agreement with an earlier study [41], showed that the lipid consists of 58% phospholipids, 38% cholesterol and 3.6% glycosphingolipids; the major phospholipids being phosphatidylcholine (22%), phosphatidylethanolamine (31%), phosphatidylserine (13%), phosphatidylinositol (4%) and sphingomyelin (27%). Such a heterogeneous lipid mixture would not be expected to undergo any gel formation above 9 °C.

In this paper we have demonstrated that a combination of FT-IR, second-derivative and ^1H–^2H exchange methods can be used to obtain structural information about the lipid and protein components of a large membrane-bound enzyme. Additionally, information can be readily obtained about the effects of ligands. Other spectroscopic techniques, such as circular dichroism and NMR, which do not require probe molecules are not readily applicable to a large membrane-bound enzyme; circular dichroism suffers from light-

scattering problems, whereas NMR is restricted by line-broadening effects and the requirement of relatively large amounts of material.

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