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CONFORMATIONAL AND MOLECULAR RESPONSES TO pH VARIATION OF THE PURIFIED MEMBRANE ADENOSINE TRIPHOSPHATASE OF *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

A preparation of ATPase from the membranes of *Micrococcus lysodeikticus*, solubilized and more than 95 % pure, showed two main bands in analytical polyacrylamide gel electrophoresis. They did not correspond to isoenzymes because one band could be converted into the other by exposure to a mildly alkaline pH value. The conversion was paralleled by changes in molecular weight, circular dichroism and catalytic properties. Denaturation by pH at 25 °C was followed by means of circular dichroism, ultracentrifugation and polyacrylamide gel electrophoresis. A large conformational transition took place in the acid range with midpoints at about pH = 3.6 ($I = 10^{-4}$ M), 4.3 ($I = 0.03$ M) and 5.3 ($I = 0.1$ M). The transition was irreversible. Strong aggregation of the protein occurred in this range of pH. The final product was largely random coil, but even at pH 1.5 dissociation into individual subunits was not complete. However, partial dissociation took place at pH 5 ($I = 0.028$ M). At this pH value the enzyme was inactive, but 20–30 % of the activity could be recovered when the pH was returned to 7.5.

In the alkaline region the midpoint of the transition occurred near pH = 11 ($I = 0.028$ M). The pK of most of the tyrosine residues of the protein was about 10.9. The unfolding was irreversible and the protein was soon converted into peptide species with molecular weights lower than those determined for the subunits by gel electrophoresis in the presence of sodium dodecyl sulphate. Conventional proteolysis did not account for the transformation.

INTRODUCTION

The energy-transducing ATPases (EC 3.6.1.3., 3.6.1.4) from mitochondria, chloroplasts and bacterial plasma membranes are very complex multi-subunit proteins whose structure and function are only beginning to be understood [1, 2]. Many of the problems concerned with the function of these enzymes and their integration into the membrane might be succesfully approached if a pure ATPase preparation

could be dissociated reversibly into its constituent subunits. The ATPase from the membranes of *Micrococcus lysodeikticus* has been solubilized in the absence of detergents [3] and purified to homogeneity [4–6]. The enzyme was morphologically and structurally very similar to those of mitochondria, chloroplasts and other bacteria [1, 2, 5–8] thus affording a good model to study this type of protein. Unfortunately, the attempts to carry out the dissociation and reconstitution for the subunits by methods similar to those used on aldolase [9] or on a number of multi-subunit enzymes [10] failed. Therefore we started a systematic study of the environmental conditions required for the reversible and irreversible dissociation of the ATPase. This paper presents the effect of the pH, and to a smaller extent that of ionic strength, on the conformation and state of aggregation of the ATPase from *M. lysodeikticus*. We show that total dissociation into subunits occurred together with or even following destruction of the tertiary and secondary structures of the constituent subunits. In some instances denaturation also involved degradation of the primary structure of the subunits.

A considerable activation of the ATPase activity at moderately alkaline pH-values was observed, together with apparent changes in the behaviour of the enzyme. A preliminary account of this work has been presented (9th FEBS Meeting, 1974, Budapest, Abstracts, p. 236).

MATERIALS AND METHODS

Chemicals

ATP was from PL Biochemicals (Milwaukee, Wisconsin, U.S.A.) and [γ-^{32}P] ATP from the Radiochemical Centre (Amersham, Bucks., U.K.). The radioactive nucleotide had a specific activity of 1–2 Ci/mmol, depending on the batch. Epi-androsterone was purchased from Roussel-Jouan (Paris). Azocoll was from Calbiochem and vitamin-free casein from Nutritional Biochemicals Co. Phenyl methyl sulphonyl fluoride was a product of Serva Feinbiochemica. Poly-L-glutamic acid from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals were of the best quality commercially available.

Enzyme assay

ATPase activity was measured by a modification of methods already described [11, 12]. The standard assay mixture contained in a final volume of 0.5 ml: 2 μmol of [γ-^{32}P]ATP ($1 \cdot 10^5$–$2 \cdot 10^5$ cpm/μmol), 2 μmol of $CaCl_2$, 4 μg of ATPase and Tris · HCl buffer pH 7.5 to a final concentration of 30 mM. The mixture was incubated for 3 min at 37 °C, then the reaction was stopped by adding 0.5 ml of a suspension of active charcoal (100 mg/ml) in potassium chloride solution (0.2 M) adjusted with HCl to a pH value of 1.8. The charcoal was then immediately spun down ($4000 \times g$, 20 min, 4 °C) and the radioactivity of aliquots (0.1 ml) of the supernatant determined.

In other cases the assay was identical except that between 20 nmol and 8 μmol of radioactive ATP and equimolar amounts of $CaCl_2$ were used. The buffers employed, all at a final concentration of 30 mM, were as follows: pH 5.0, sodium acetate; pH 5.5–7.0, sodium cacodylate, adjusted with HCl; pH 7.5–9.0, Tris · HCl; pH 9.5–10.5, glycine/NaOH. The reaction was not linear with time beyond the first 3 min. In other experiments the pH-stat technique was employed using a Radiometer assembly

(pH-meter pHM 26, titrator 11, ABU 12 burette, SBR 2c recorder and GK 2320 C semimicrocombination electrode). The reaction mixture was maintained at 37±0.1 °C by circulating water through a jacket in the reaction vessel by means of a constant temperature bath (Lauda K2RD, G.F.R.). The electrode was standardized at 37 °C using Beckman standard buffer pH 7.0. The equivalence of phosphate liberated with NaOH added (8–10 mM) at different pH values was derived from the curves shown by Dyson and Noltman [13]. The reaction mixtures were 30 mM with respect to KCl.

Protein concentration

The concentration of a pure ATPase solution was determined by the number of fringes produced in the analytical ultracentrifuge when it was centrifuged in a 12 mm synthetic boundary double-sector capillary cell against the same buffer in which the protein was dissolved. A value of 40 fringes for a concentration of protein of 10 mg/ml was used. The concentration thus determined was used to obtain a specific extinction coefficient of $E^{1\%}_{1\,cm} = 6.9$ at 276 nm, which was subsequently used in estimations of the concentration of the pure enzyme.

Circular dichroism spectra

Circular dichroism (CD) spectra were measured with a Roussel-Jouan 185 dichrograph II (Paris, France), fitted with a xenon arc lamp and calibrated with epiandrosterone and poly-L-glutamic acid. The 10^{-5} ΔE/mm sensitivity scale was generally used, together with timeconstants of 4 or 2 s. The cell-holder temperature was kept at 25±0.1 °C with the help of a constant-temperature bath. The values of the dichroic absorption in a 1 cm light path, ΔE, were converted to mean residue molecular ellipticity, $[\Theta]$, by means of the relationship

$$[\Theta] = 3298\ \Delta E/c \text{ deg} \cdot \text{cm}^2/\text{dmol},$$

where c was the mean residue concentration in mol/litre. A value of 109 was used for the mean residue weight, based on the reported amino acid analysis [5] and the determinations of tyrosine and tryptophan in this paper. Measurements and adjustments of pH were performed as described below. The presence of light scattering was checked by recording ultraviolet absorption spectra. Any drift in the base line caused by this or any other reason was detected in the dichroism in the region 250–270 nm and was corrected by difference.

Absorption spectra

Absorption spectra and difference spectra were measured with a Cary 16 S recording spectrophotometer, using slit widths of 0.2–0.3 mm and a pen period of 1 s. Jacketed cell-holders were used and their temperature kept at 25±0.1 °C by means of a constant temperature bath.

When cuvettes of 1 cm light-path were used the pH value of the protein solution was measured and adjusted inside the cuvette. Adjustment was performed by addition of HCl or KOH (Carlsberg micropipettes) of the appropriate concentrations so as to avoid dilutions higher than 0.5 %.

pH measurements

pH measurements were made with a pH-meter pHM 26 from Radiometer

(Copenhagen) fitted with a combination semimicroelectrode (Metrohm AG, Herisau, Switzerland), adequately standardized before measurements [14].

Measurement of radioactivity

Radioactivity from ^{32}P was measured in a Nuclear Chicago Mark II liquid scintillation counter. The scintillator was a Bray's cocktail composed of naphtalene (60 g), 2-(4'butylphenyl)-5-(4''-biphenyl)-1, 3, 4-oxadiazole (butyl-PBD, CIBA) (5 g), ethyleneglycol (20 ml) and methanol (100 ml) dissolved in dioxan to a final volume of 1 l. Radioactive phosphate was counted with an efficiency of 65 %.

Analytical ultracentrifugation

The analytical ultracentrifuge was a Spinco model-E (Beckman Inst., Palo Alto, Calif., U.S.A.) equipped with Rayleigh interference optics and a RTIC temperature control unit. Molecular weights were determined by the method of Yphantis [15] at about 14 °C in a double-sector cell as described before [5]. The partial specific volume (0.74 ml/g) was calculated from the amino acid composition as described [16]. Sedimentation was performed at rotor speeds ranging from 10 000 to 56 000 rev./min. The density of the solutions was found in several cases to be equal to that of water at the same temperature and this was assumed to be generally valid, except for the experiment at high salt concentration (2.67 M KCl) where density was 1.132 g/cm^3.

Polyacrylamide gel electrophoresis

Electrophoresis at pH 8.5 was carried out following the general procedure described without stacking gels [5]. Separating gels with 7 % acrylamide and 0.17 % *N,N'*-methylenebisacrylamide were employed but in some instances 10 % acrylamide, 0.24 % *N,N'*-methylenebisacrylamide were used. Gels in 5×0.6 cm (short) or 12×0.6 (long) columns were polymerized in the dark and electrophoreses were run at different pH values in buffers of a molarity as close as possible to 30 mM, as used in enzyme assays, ultracentrifugation and spectroscopy. The buffers used both in the gel and in the electrode chambers were the following: 30 mM glycine titrated with NaOH to pH 9.5 (about 6 mM NaOH) and to pH 10.5 (approx. 12 mM NaOH); 20 mM sodium acetate/10 mM acetic acid, pH 5.0±0.1; 7.5 mM sodium acetate/22 mM acetic acid, pH 4.2±0.1; 25 mM glycine adjusted to pH 3.0 with HCl; and 25 mM sodium phosphate, pH 11.2. For electrophoresis in the last two buffers the gels were polymerized at pH values of 5.0 and 10.5 respectively, and pre-run without samples for 1–3 h with the corresponding buffer of the desired final pH value. Electrophoreses were run in water-cooled tanks (long gels) or at room temperature for 35 min–2 h at 3.5 (short gels) or 5 (long gels) mA/column. The samples containing 10 % glycerol were layered on the gels with bromophenol blue as tracking dye. Except at pH 3 and 4 migration was towards the anode. Electrophoresis in dissociating conditions was carried out in the sodium dodecyl sulphate-alkaline pH (8.5±0.2) system as described by Andreu et al. [5]. Proteins were stained with R 250 Coomassie brilliant blue following the procedure of Fairbanks et al. [17]. After staining, the gels were scanned at 575 nm with a 2400 Gilford Spectrophotometer equipped with the model 2419 S linear transport system.

Electron microscopy

The microscope was a JEM 100 B (JEOL Ltd., Tokyo, Japan). Copper grids coated with a thin carbon film (50–70 Å) were used and the preparation negatively stained with 1 % ammonium molybdate in 2 % ammonium acetate, pH 7.2 [18].

Assay of proteolytic activity

Possible intrinsic or contaminant proteolytic activity at alkaline pH values of purified *M. lysodeikticus* ATPase was assayed with either Azocoll (5 mg/1.5 ml of 30 mM glycine/NaOH (pH 10.0); 50 mM sodium bicarbonate/NaOH (pH 11.0); or 50 mM Na_2HPO_4 titrated to pH 11.2 with NaOH) or 1 % (w/v) casein in glycine or bicarbonate buffers as substrates. The assays were carried out at 25 °C or 37 °C for 1–3 h using 100 μg pure protein and maintaining a constant pH during the incubation by the pH-stat assembly. After sedimentation of the Azocoll by centrifugation at 10 000 $\times g$ for 30 min, the absorbance of the supernatant at 520 nm was measured. When casein was used as substrate, undigested protein was precipitated with 5 % (w/v) trichloroacetic acid and the $E^{1\,cm}_{280\,nm}$ of the supernatant measured.

RESULTS

The ATPase preparation and its stability

The solubilization and purification of the membrane-bound ATPase of *M. lysodeikticus* (NCTC 2665) have already been described [3–5]. The preparation used in this work had a specific activity of 6.5–7.0 μmol/mg of ATPase per min assayed under standard conditions as described in the Methods. It contained α, β, γ and δ subunits in the proportion 3 : 3 : 0.7 : 1 respectively, calculated from the areas under the 575 nm scannings of sodium dodecyl sulphate polyacrylamide gels stained with Coomassie brilliant blue [5]. As a control of the purity of the ATPase, electron microscopy of the negatively stained enzyme was carried out. A picture of the preparation used in this work is shown in Fig. 1. The particles, about 100 Å across, looked very homogeneous and had a similar appearance to those previously reported for *M. lysodeikticus* [18] and mitochondrial ATPase [19]. Although they looked roughly hexagonal the quality of the micrographs did not warrant further analysis.

When the polyacrylamide gel electrophoresis pattern of the ATPase was obtained in columns 5 cm long, it consisted of a single band with a relative mobility of about 0.26. However, when the protein was applied to 12 cm-long gels the pattern consisted of three bands of relative mobilities 0.23, 0.26 and 0.28 (Fig. 2). They have been numbered 1, 2 and 3 in order of increasing relative mobility. With fresh samples the slowest (band 1) predominated, whereas long term storage at −20 °C (up to 24 months) slowly converted the enzyme into band 2 and, to a much lesser extent, into band 3 (Fig. 2). As the conversion into bands 2 and 3 proceeded the simultaneous appearance of two bands with relative mobilities of about 0.45 and 0.46 (bands 4 and 5, Fig. 2) was observed. In electrophoresis in 10 % polyacrylamide gels each of bands 4 and 5 was resolved into at least three components. When the proportion of bands 2 and 3 increased noticeably (Fig. 2, 1 year old sample) the enzyme had lost about 70 % of its activity. Repeated (5–7 times) thawing and freezing (−20 °C) of an enzyme solution (0.2–10.0 mg/ml) had a similar effect. Therefore, the enzyme solution in 50 mM Tris sulphate buffer pH 7.5, at a concentration of 10 mg/ml, was divided in

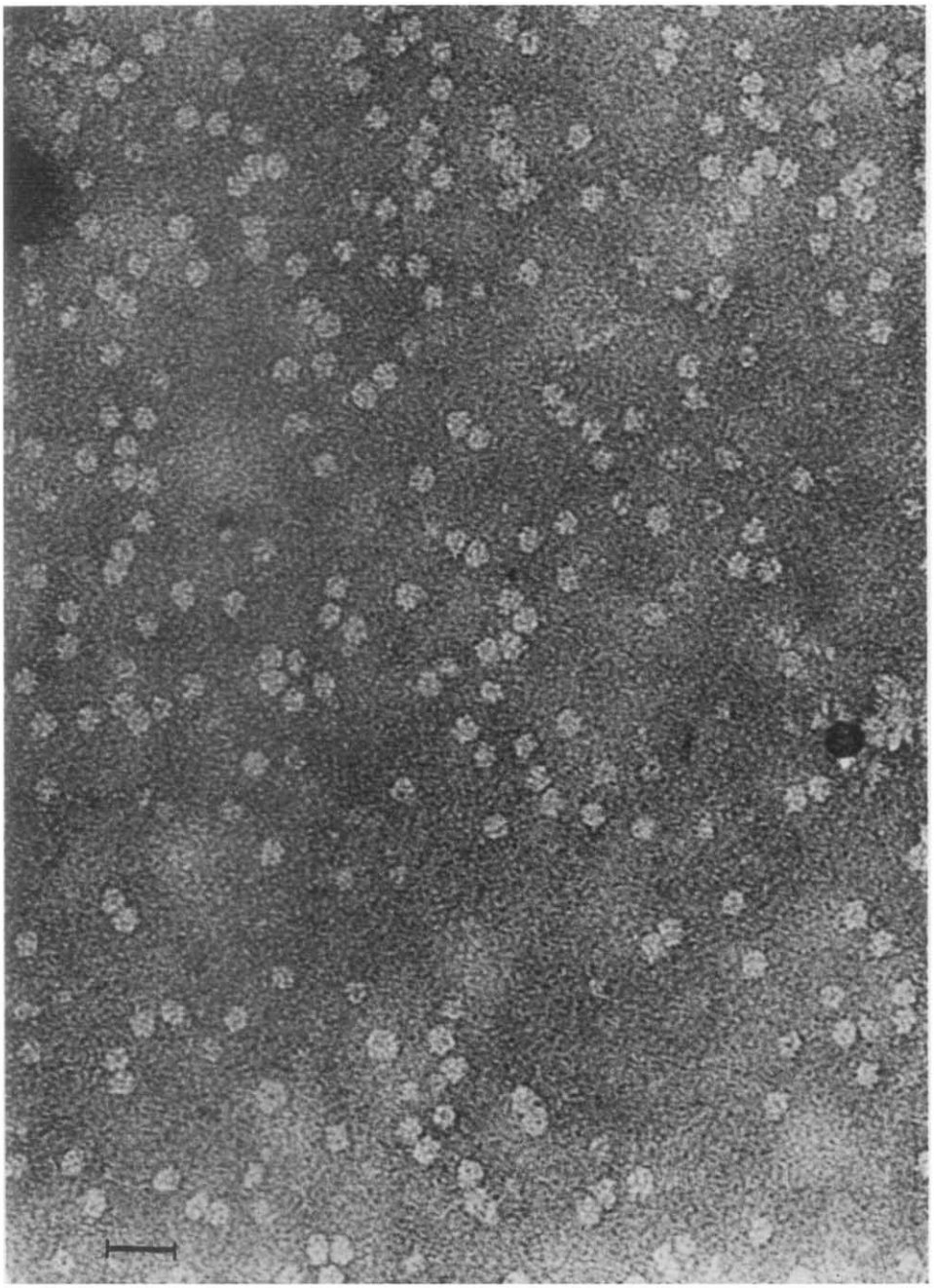

Fig. 1. Purified *M. lysodeikticus* ATPase negatively stained with ammonium molybdate. Magnification × 300 000. The bar represents 300 Å.

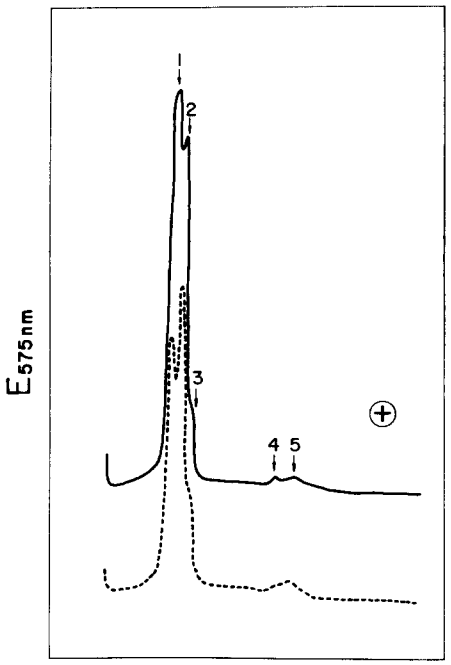

Fig. 2. Densitometric scan at 575 nm of polyacrylamide gel patterns of ATPase. The traces belong to: —, protein stored 5 months at −20 °C; – – –, stored for 1 year. The bands had the following relative mobilities: 1, 0.23; 2, 0.26; 3, 0.28; 4, 0.45; 5, 0.46. Migration was in all cases towards the anode; 40 μg of protein were applied.

lots of 50 μl, and each was used in less than one week, avoiding its thawing more than 3 times. Most of the work reported was performed with a protein preparation 3 to 6 months old. Under these conditions the shape and intensity of the CD and ultraviolet absorption spectra as well as the enzymic activity were not affected. Bands 2, 4 and 5 seem to accompany band 1 even when the enzyme preparation has just been obtained (see also ref. 4). In that case bands 4 and 5 might be so faint as to escape easy detection, band 3 might or might not be present and bands 1 and 2 come together to give an apparent single broad band. Therefore, it was extremely difficult (if possible) to obtain an enzyme preparation consisting of a single component. All we could do was to describe the proportions of the three forms of the enzyme in the preparation used in this work. These proportions can only be qualitatively estimated from the scanning of the stained gels (Fig. 2) due both to strong overlapping of the bands and to the possibility that they might not stain with equal intensity.

Ultraviolet absorption and circular dichroism at neutral pH

The ultraviolet spectrum of the pure ATPase, after correction for a small amount of scattering present [20], showed a maximum at 275–276 nm, three small shoulders at about 258, 265 and 269 and a pronounced shoulder near 290 nm (Fig. 3). The scattering-corrected extinction was directly proportional to the concentration of protein in the range from 0.03 to 10.0 mg/ml. The specific extinction of the ATPase, estimated as an average from 9 independent experiments, was $E^{1\%}_{1\,cm} = 6.67 \pm 0.1$ at

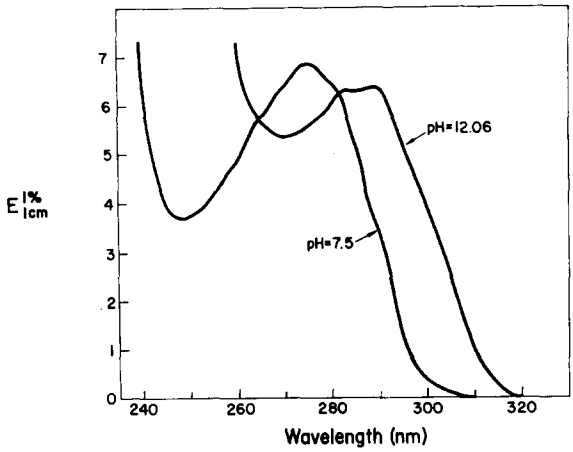

Fig. 3. Ultraviolet spectrum of *M. lysodeikticus* ATPase. The curves shown were the average of 10 (pH 7.5) and 3 (pH 12.0) spectra, respectively. The concentration of protein ranged from 0.03 to 10.0 mg/ml and the light path of the cuvettes from 0.1 to 1.0 cm. The neutral spectra were recorded in Tris · SO_4 buffer pH 7.5 (20–50 mM). Temperature, 25 °C.

280 nm and $E^{1\%}_{1\,cm} = 6.93 \pm 0.1$ at 276 nm. Using a value of 350 000 for the molecular weight [5], values of $\varepsilon\,276 = 242\,000 \pm 3500\ M^{-1} \cdot cm^{-1}$ and $\varepsilon\,280 = 233\,000 \pm 3500\ M^{-1} \cdot cm^{-1}$ were calculated.

The far ultraviolet CD spectrum of the ATPase at pH 7.5 is shown in Fig. 4. It had two negative extrema at 221–222 ($[\Theta] = -8500 \pm 300$) and 209–210 nm ($[\Theta]$

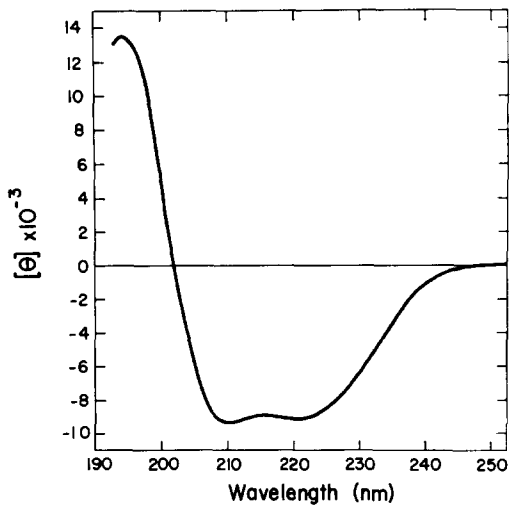

Fig. 4. Circular dichroism spectrum of *M. lysodeikticus* ATPase. The curve shown was the average of 15 spectra, each obtained in duplicate. The concentration of protein ranged from 0.03 to 2.01 mg/ml and cuvettes of light path from 0.01 to 1.0 cm were used. All spectra were obtained in Tris · sulphate buffer pH 7.5 (10–50 mM). $[\theta]$ is the mean residue ellipticity (deg · cm^2/dmol). Temperature, 25 °C.

$= -8800 \pm 600$) crossover at 202 nm and a positive maximum at about 194 nm ($[\Theta] = +13900 \pm 1600$). The general shape of the spectra indicated the presence of a certain amount of α-helix, as well as some β-structure. From the values of the ellipticity at 208 nm [21] the content of α-helix was calculated as about 18 % of the residues. Using the values of ellipticity in the range 208–240 nm according to Greenfield and Fasman [21], the estimated structural composition of the protein was about 20 % α-helix and 29 % β-structure. If the method of Saxena and Wetlaufer [22] was used, those values became 24 % α-helix and 28 % β-structure. The values of CD at 210 and 222 nm were linear with concentration in the range from 0.02 to 2.0 mg/ml. The far ultraviolet spectrum was not affected by up to 67 mM $MgCl_2$ or 16 mM sodium phosphate.

The spectrum in the near ultraviolet was featureless and very weak, showing a broad positive band with maximum value near 276 nm ($[\Theta]276 = 10$).

Influence of ionic strength at neutral pH value

It was desirable to test the effect of pH on the properties of the ATPase under conditions where the influence of other variables was known. Therefore we examined the influence of ionic strength on the CD of the protein at concentrations ranging from 0.02 mg/ml to 0.2 mg/ml and ionic strengths from $1 \cdot 10^{-4}$ M to 3 M in Tris sulphate buffer (pH 7.5) or that buffer containing KCl. No change was observable either in the shape or the magnitude of the CD spectra in the far ultraviolet. Under similar conditions the enzyme activity changed considerably, showing an optimum at an ionic strength of 0.030–0.035 M.

Tyrosine and tryptophan contents

The contents of these aromatic amino acids had been previously estimated, although subjected to a large error [5]. Because of their important contribution to the optical properties of the ATPase and their possible involvement in the stability and enzymic activity of the protein, we decided to estimate the contents of these residues with greater accuracy.

The tyrosine to tryptophan ratio was determined from scattering corrected ultraviolet spectra at neutral or basic pH values in the presence or absence of 6 M guanidinium hydrochloride. From the alkaline spectrum (pH 12.06, Fig. 3), values of $\varepsilon 294.4 = 193\,400$ and $\varepsilon 280 = 208\,200$ $M^{-1} \cdot cm^{-1}$ were obtained, and application of the method of Goodwin and Morton [23] gave 54 tyrosine and 23 tryptophan residues per 350 000 g of ATPase. From the difference spectrum of the protein in alkali (pH 11.9) against the protein at a neutral pH, values of $\Delta \varepsilon 293 = 157\,000$ and $\Delta \varepsilon 242 = 723\,000$ $M^{-1} \cdot cm^{-1}$ were obtained. By using the values of $\Delta \varepsilon_{max}$ for *N*-acetyltyrosine given by Donovan [24], the number of mol of tyrosine per 350 000 g of protein was estimated as 65 at both wavelengths.

The neutral spectrum in 6.0 M guanidinium hydrochloride gave values of $\varepsilon 288 = 126\,500$ and $\varepsilon 280 = 204\,000$ $M^{-1} \cdot cm^{-1}$. For these values the method of Edelhoch [25] afforded 21 mol of tryptophan and 56 of tyrosine per mol of protein. Finally, the alkaline difference spectrum of the ATPase in 6 M guanidinium hydrochloride had $\Delta \varepsilon 295 = 150\,500$ and $\Delta \varepsilon 300 = 144\,000$ $M^{-1} \cdot cm^{-1}$. Using the corresponding values for tyrosine given by Edelhoch [25] the molar contents of that amino acid were estimated as 61 (295 nm) and 63 (300 nm) residues per mol of

protein. Therefore, the ATPase had 60±4 mol of tyrosine and 22±2 mol of tryptophan per mol of enzyme (350 000 g). These values were not in very good agreement with the amino acid analysis reported earlier [5] but fell within the large deviations reported. Using values of $\varepsilon 280 = 5690$ for *N*-acetyltryptophan amide and $\varepsilon 280 = 1300$ for glycyltyrosylglycine in 6 M guanidinium hydrocloride at neutral pH [25], a value of $\varepsilon 280 = 203\,600\ M^{-1} \cdot cm^{-1}$ could be predicted for the molar extinction of unfolded ATPase in 6 M denaturant. This was in very good agreement with the experimental value ($\varepsilon\ 280 = 204\,000\ M^{-1} \cdot cm^{-1}$).

Stability of the enzymic activity as a function of pH

The stability to pH of the ATPase activity was examined by incubating at the desired pH value an enzymic solution (35 μg/ml) in the absence of substrate at $I =$ 0.028 and 25 °C for 1 h, then returning to pH 7.5, adding substrate and assaying the activity as usual. The enzyme was quite stable in the pH range from 7.5 to 10.0 but lost about 50 % of its activity by pre-incubation at pH 10.6 (Fig. 5). Enzymic activity was totally abolished at pH 11 where a large unfolding transition took place (see below).

Fig. 5 also shows a plot of the activity of the enzyme (see below) against pH value. The enzyme shows an optimum from about pH 8.5 to 9.5, decaying quite sharply on both sides of this range.

Circular dichroism dependence on the pH value

The influence of the pH value on the CD of the ATPase was examined at ionic strengths of 10^{-4}, 0.03 and 0.1 M. In all cases spectra were obtained as well as values of CD at 222 and 210 nm. Usually spectra were recorded at 10, 20 and 40 min intervals after adjusting the pH. When cuvettes of 1 cm light-path were used, the pH

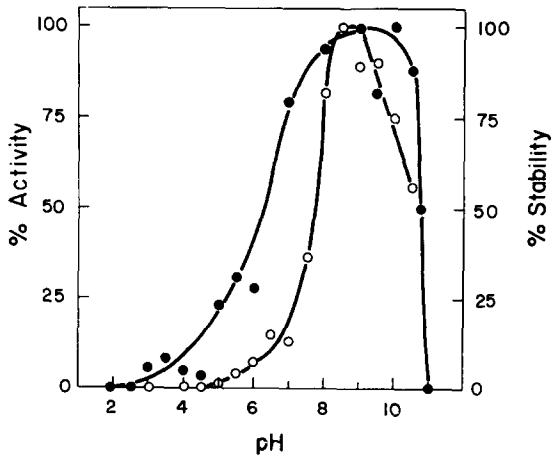

Fig. 5. Enzymic activity and stability of the enzymic activity as a function of pH. ATPase activity (○–○) was assayed under standard conditions (see Methods). To test the stability (●–●) the protein was incubated alone at the desired pH value for 1 h at 25 °C, then the pH was adjusted to 7.5 in Tris · HCl buffer and the activity assayed as above. None of the buffers used were inhibitory, relative to each other.

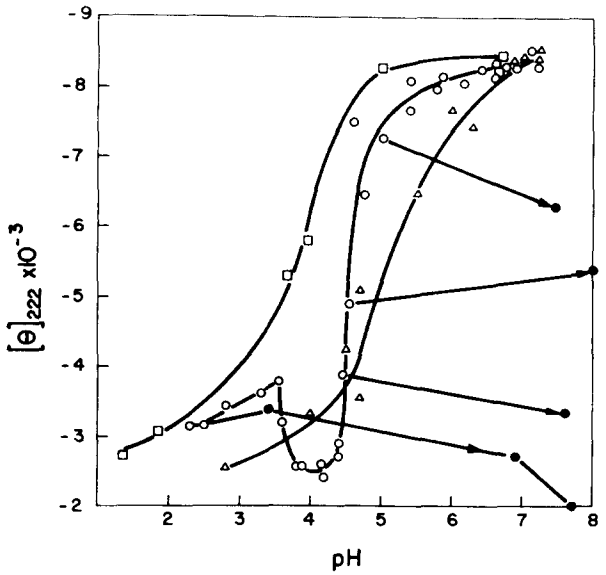

Fig. 6. Dependence of the $[\theta]_{222}$ on the pH value in the acid range. The protein concentration was 0.03 to 0.08 mg/ml, with light paths from 1 cm to 0.2 cm and dichrograph sensitivity of $1 \cdot 10^{-5}$ ΔE/mm. The ionic strength was 10^{-4} (□–□), 0.028 (○–○) and 0.105 (△–△). $[\theta]_{222}$ is mean residue ellipticity at 222 nm (deg · cm^2/dmol). Temperature, 25 °C. Filled circles represent back-titrations.

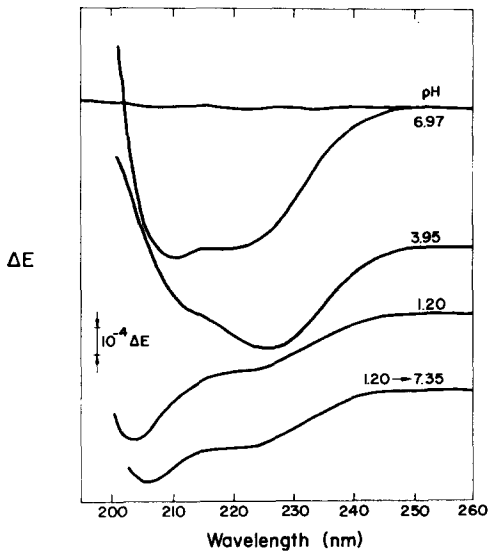

Fig. 7. Circular dichroism of the ATPase at acidic pH values. The protein concentration was 0.02 mg/ml and the light-path 1 cm. Ionic strength was 0.005 M, and temperature, 25 °C. ΔE is $E_L - E_R$, the difference in absorption for left and right hand circularly polarized light.

was measured inside the cell. In all cases the pH was measured before and after recording the CD of the sample and differences were generally better than ± 0.1 unit. When a large, time-dependent, conformational transition occurred it was followed with time at 222 nm until a stable value of CD was reached. Possible drifts in the baseline due to aggregation or other reasons were corrected by recording the CD at 270 nm. Drifts were rare and in no case exceeded $5\text{–}6 \cdot 10^{-5}$ ΔE.

(a) *Acidic range.* The dependence of the CD on the pH in the acid region is shown in Fig. 6. If $[\Theta]_{210}$ was chosen instead of $[\Theta]_{222}$ the result was similar. An apparently single transition is seen, the midpoint of which was closely dependent upon the ionic strength. It occurred at pH values of about 3.6, 4.3 and 5.3 at ionic strengths of 10^{-4}, 0.028 and 0.1 M, respectively. At a pH value near that at which the transition took place strong aggregation could be detected in the ultraviolet or in the CD spectra (Fig. 7). At an ionic strength of $I = 0.028$ M and a pH value near 4.2, some of the protein came out of solution. This was reflected in the value of $[\Theta]_{222}$, which decreased to a minimum at that pH value, to increase at lower pH values when aggregation was reversed to a large extent. At those lower pH values, the CD spectra resembled that of proteins with a large amount of random coil conformation (Fig. 7, pH 1.2). As shown in Fig. 6, the transition was irreversible. Although refolding took place (Fig. 7) this was not a return to the native conformation. Furthermore, the protein came out of solution to a large extent when the pH was returned to neutral

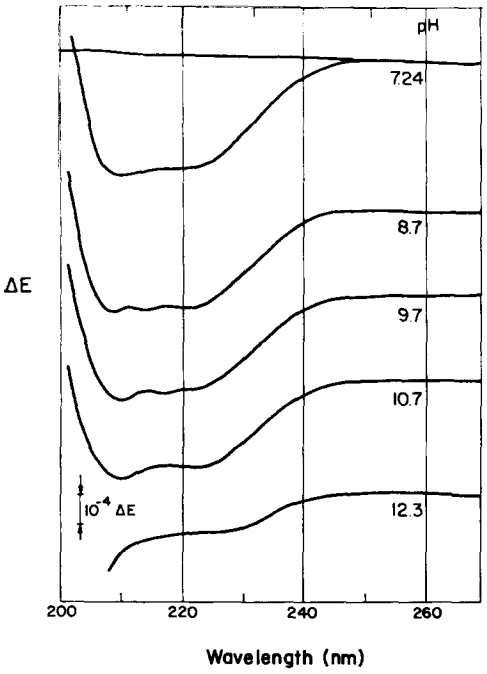

Fig. 8. Circular dichroism spectra of the ATPase at alkaline pH values. The spectra were obtained at a protein concentration of 0.08 mg/ml and 0.2 cm light-path. The ionic strength was 0.028 and temperature 25 °C. ΔE is $E_L - E_R$, the difference in absorption for left and right hand circularly polarized light. Temperature, 25 °C.

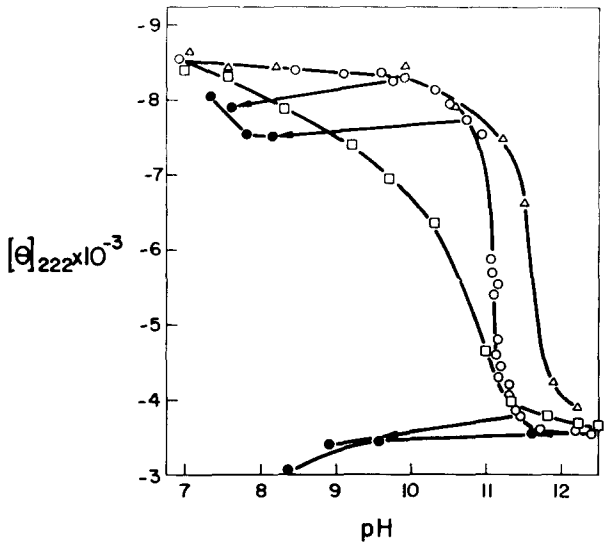

Fig. 9. Dependence of the $[\theta]_{222}$ on the pH value in the alkaline region. For experimental conditions see legend of Fig. 6. The ionic strength was: 10^{-4} (△-△): 0.028 (○-○); and 0.105 (□-□). Temperature, 25 °C; (●) represents back titrations.

values, as shown by the scattering present in the ultraviolet spectra and the strong decrease in the CD signal.

(b) *CD in the range of pH 7.0 to 10.7.* In this range small changes in the shape of the CD spectrum were observed which were not adequately reflected in the $[\Theta]_{222}$. These included an increase in the dichroism at 210 nm relative to that at 222 nm, as well as the appearance of a hump near 215 nm (Fig. 8). At an ionic strength of $I =$ 0.028 M, these changes started at a pH value near 8.7 and as the pH was raised to about 10.1–10.4 the hump was eliminated although a slight increase in the value of $[\Theta]_{210}$ relative to $[\Theta]_{222}$ persisted (Fig. 8). At $I = 0.1$ these fine changes occurred in the range of pH from about 8.5 to 9.6 and the lowest ionic strength (10^{-4} M) the hump was still present at pH 10.5. The decrease in the value of $[\Theta]_{222}$, although small, was irreversible. This is shown in Fig. 9 for $I = 0.028$ M and similar results were obtained at the other ionic strengths. These changes did not appear to be related to denaturation because enzyme activity was unaffected or even stimulated (see Fig. 5).

(c) *Alkaline range.* The dependence of the CD on the pH in the alkaline region was also followed at ionic strengths of 10^{-4}, 0.028 and 0.1 M. From a plot of $[\Theta]_{222}$ or $[\Theta]_{210}$ against pH (Fig. 9), the pH values obtained for the midpoint of the transition were 11.5, 11.1 and 10.8 in order of increasing values of ionic strength and the final product had a dichroism reminiscent of the random coil.

The behaviour of tyrosine residues during alkaline titration was examined by ultraviolet difference spectroscopy. The scattering corrected alkaline difference spectrum of the ATPase was typical of ionized tyrosine side chains [24]. A plot of the difference at its maximum (i.e., 295 or 242 nm) against pH gave a titration curve for the tyrosine residues of native ATPase (Fig. 10), the pK of which was about 10.9. If

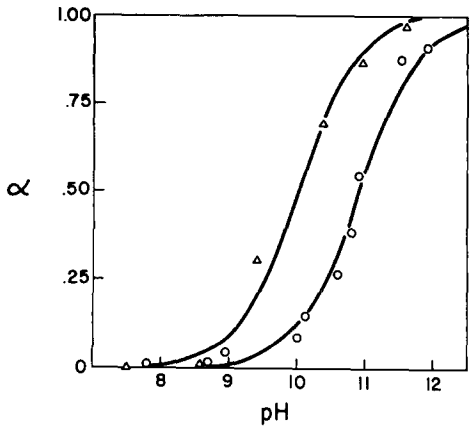

Fig. 10. Spectrophotometric titration of the tyrosine residues of the ATPase. The fraction of tyrosine residues ionized (α) was determined at an ionic strength of 0.028 M (○–○) and in 6 M guanidinium hydrochloride (△–△) as the ratio between the difference in absorption at a given pH value and the maximum difference possible. The concentration of protein was 0.08 mg/ml, and the temperature, 25 °C. The solid lines represent the theoretical titration curves for groups with pK values of 10.0 and 10.9, respectively.

the protein was first dissolved in 6 M guanidinium hydrochloride and then titrated, the pK obtained was near 10.0 (Fig. 10). This latter value compares very well with that reported by Edelhoch [25] for tyrosine in small molecular weight model compounds. The pK of the tyrosine residues in the native protein suggested that the great majority of them were not freely accessible to the solvent until dissociation and/or unfolding occurred. As shown in Fig. 9, the alkaline transition was also irreversible and, furthermore, very small values for the molecular weight were obtained soon after unfolding (see below).

Kinetics of the acid transition

The rate at which the acid transition occurred was measured by recording continuously the circular dichroism at 222 nm. First-order rate constants, k_1 were computed from the equation

$$\text{In}\,\frac{\Delta E_t - \Delta E_\infty}{\Delta E_0 - \Delta E_\infty} = -kt \qquad (1)$$

where ΔE_t, ΔE_0 and ΔE_∞ are the dichroic absorption at time t, at zero time and at infinite time, i.e., after completion of the reaction. Straight lines were adjusted by means of a least squares program, and Table I shows the values of k at several pH values. The values of ΔE_t used varied from $t = 0$ up to that time at which about 95 % of the transition had occurred. The good or excellent correlation coefficient (R) shown in Table I indicated that the rate of transition followed first-order kinetics. Near pH 4 the decrease in CD at 222 nm corresponded with the appearance of scattering in the ultraviolet spectrum as shown in Table I, where one of the values of k (pH = 4.3) was estimated from the values of the apparent absorbance at 238 nm. The aggregation reflected by this appearance of scattering was largely reversed at lower pH values (i.e., pH 3.6).

TABLE I

RATE OF ACID - DENATURATION OF *M. LYSODEIKTICUS* ATPase

The protein solution (0.03–0.08 mg/ml) was in 28 mM KCl, 0.5 mM Tris–HCl, pH 7.5. The pH, adjusted by adding 3.8 M HCl, was measured at the end of the reaction. *R* was the correlation coefficient in least squares adjustment.

pH	k ($s^{-1} \times 10^3$)	R
3.44	8.76	0.96
3.49	11.16	0.91
3.67	1.96	0.95
3.97	3.32	0.99
4.3	1.14	0.999 (ultraviolet scattering)
4.36	1.01	0.99
4.39	0.87	0.999
4.59	0.45	0.99

Kinetics of the alkaline transition

On the alkaline side in 28 mM KCl and 25 °C the change of CD at 222 or 210 nm beyond pH 10.9 was composed of a very fast decrease, which could not be studied in the dichrograph, followed by a slow change to give a final product with a large amount of random coil. The rate constants for the slow reaction were $7.4 \cdot 10^{-4}$ s^{-1} at pH 11.1 and $3.14 \cdot 10^{-3}$ s^{-1} at pH 11.5. The ionization of tyrosine residues was also very fast and probably related to the initial decrease in CD.

Molecular weight dependence on the pH value

The dependence of the molecular weight of the ATPase on pH was examined at ionic strength values of 0.03–0.06. The high speed equilibrium method [15] was used as described by Roark and Yphantis [26]. With the exception of the experiments performed at pH 7.5 and pH 5.0, the plots of ln j against r^2 were composed of two straight-line portions (Fig. 11). This, of course, was an indication of heterogeneity and the values of molecular weight recorded in Table II are weight averages. At pH 5.0 where a large transition was about to occur (Fig. 6), the protein was already partly dissociated as reflected in the value of molecular weight (Table II). At pH 4.2 dissociation perhaps continued, but it was completely masked by precipitation of the protein. After separation of the precipitate the protein remaining in solution was formed predominantly by large aggregates of molecular weight higher than $3 \cdot 10^6$. If we remember the sharp decrease observed in the $[\Theta]_{222}$ (Fig. 6) or in $[\Theta]_{210}$ (not plotted) at this pH value, we may conclude that it was due mainly to protein precipitation, difficult to observe visually at the concentrations used in CD experiments (0.04 mg/ml) but quite evident at the concentrations used in the ultracentrifuge. Below pH 4.2 (i.e., at pH values of 3.1 and 1.5) these large aggregates were destroyed and CD indicated the appearance of a large amount of disordered conformation. However, even at these low pH values the protein was not completely dissociated (Table II).

In the alkaline range only small changes in the molecular weight took place until a pH value of 11.0. The slightly lower molecular weight found at pH 10.0 was clearly an average and reflected the appearance of low molecular weight species as indicated also by polyacrylamide gel electrophoresis (see below). At pH 11.0 a large

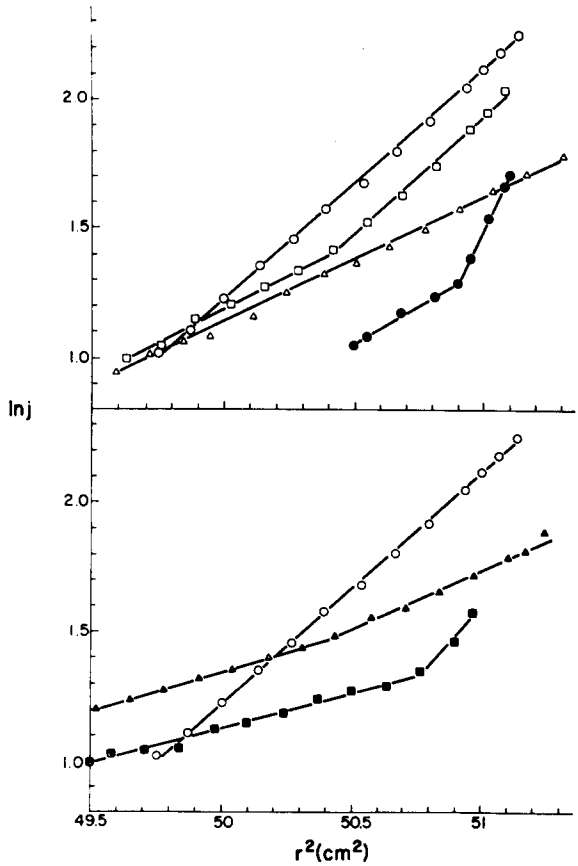

Fig. 11. Sedimentation equilibrium of ATPase at: pH 1.5, 30 000 rev./min (●–●); pH 3.15, 10 000 rev./min (□–□); pH 5.0, 10 000 rev./min (△–△); pH 7.5, 10 000 rev./min (○–○); pH 11.0, 30 000 rev./min (■–■); and pH 12.0, 30 000 rev./min (▲–▲). Other conditions as indicated in Table II.

conformational transition took place (Fig. 9) with simultaneous dissociation of the ATPase. Probably more than two species were present in the experiments at 30 000 rev./min rotor speed (Table II and Fig. 11) and some of them had an average molecular weight of 10–11 000. Because some contribution from the higher molecular weight species should be expected, the true size of the low molecular weight components must be even lower than these values. This is shown in the experiments carried out at higher rotor speed (56 000 rev./min), where average molecular weights as low as 3500 and 6800 were found. These values are much lower than those previously estimated for the subunits of the ATPase [5]. The conclusion is that the alkaline unfolding shown by optical methods occurred simultaneously with dissociation and chemical degradation of the subunits of the ATPase. This has been confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (see below).

TABLE II

DEPENDENCE OF THE WEIGHT AVERAGE MOLECULAR WEIGHT OF THE ATPase ON THE pH VALUE

The protein solution (0.5–0.7 mg/ml) was 30 mM in the following buffers: pH 3.15, glycine · HCl; pH 4.23 and 5.0, sodium acetate; pH 7.5, Tris · HCl; pH 10.0, glycine/NaOH; pH 11.0 and 12.0, sodium phosphate. The pH value 1.5 was in 0.06 M HCl. The values of the temperature of the rotor were in the range from 13 to 14 °C. A double-sector 12 mm cell was used in all the experiments.

pH	Rotor speed (rev./min)	Molecular weight	Comments
1.5	30 000	28 600	
		88 700	Two independent ex-
		29 300	periments. After 24 h
		87 600	
3.15	10 000	202 700	
		356 800	
4.23	5–10 000	$> 3 \cdot 10^6$	Protein precipitates
5.00	10 000	208 500	Two bands in gel electro-
			phoresis
7.50	10 000	345 000	
10.0	10 000	333 600	
11.0	30 000	9 700	After 30 h. Two inde-
		48 500	pendent experiments
		11 000	
		47 200	
	56 000	3 500	After 30 h
		6 800	
12.0	30 000	15 400	After 48 h
		21 400	
		13 200	After 100 h
		19 600	
	56 000	8 600	After 100 h
		11 800	

Polyacrylamide gel electrophoresis patterns as a function of the pH value

At a neutral or slightly alkaline pH (pH 7.5–8.5) the polyacrylamide gel electrophoresis patterns of the ATPase in 12-cm long gels consisted usually of three bands of relative mobilities 0.23, 0.26 and 0.28. Polyacrylamide gel electrophoresis was also performed in the range of pH values from 3 to 11. In all cases the ATPase samples were kept at the desired pH value for 30–40 min prior to its application to the gels. At pH 5 migration was still towards the anode; at pH 4.2 the protein was very insoluble and formed large aggregates that did not enter the gel. This suggested that the isoelectric point of the ATPase was in the vicinity of pH 4. At a pH value of 3 the protein probably migrated towards the cathode, but most of it was still unable to enter the gels. Fig. 12 shows the 575 nm scans of the gels at pH values of 5.0 to 11.2. At pH 5.0, two distinct bands were evident with a relative mobility near 0.17–0.19. At pH 11.2, six or seven components were present and the mobility of the most intense was about 0.23 (Fig. 12). The heterogeneity present after alkaline denaturation of the protein involved degradation of the subunits of the enzyme. This was shown by performing electrophoresis under dissociating conditions (i.e. in the presence of

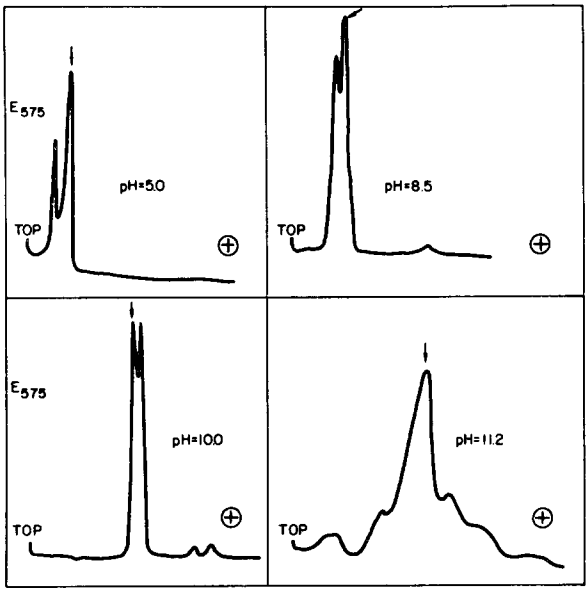

Fig. 12. Densitometric scan at 575 nm of polyacrylamide gel patterns of ATPase at several pH values. Migration was in all cases towards the anode. The relative mobilities of the most intense bands (arrow) were as follows: pH 5, 017; pH 8.3, 0.26; pH 10.0, 0.24; pH 11.2, 0.23. The amount of protein applied to the gels was 40 μg.

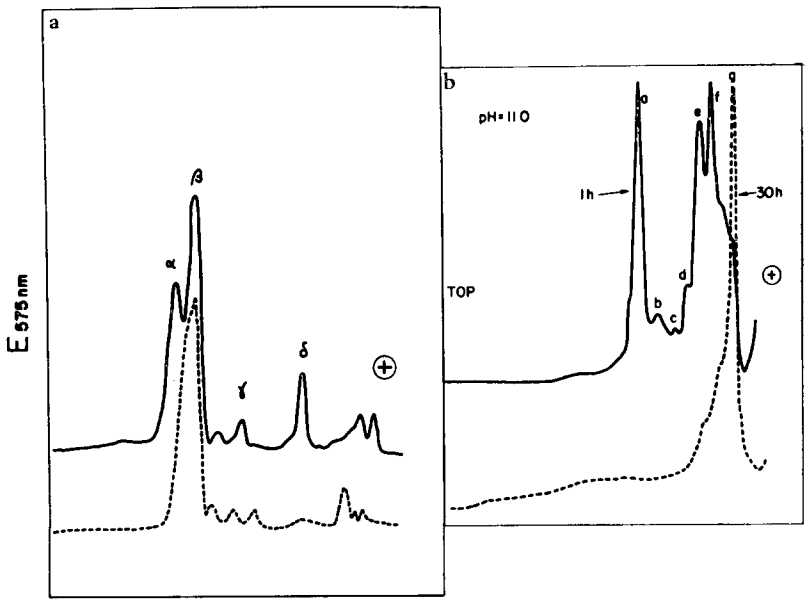

Fig. 13. Densitometric scans at 575 nm of sodium dodecyl sulphate polyacrylamide gel patterns of ATPase. (a) —, Protein control, stored from 0–5 months at −20 °C; - - -, protein exposed to pH 10.0, 3 h and then returned to pH 7.5, or thawed 7 times; 40 μg protein were applied. (b) After maintaining the protein (40 μg) at pH 11 for the time stated in the figure polyacrylamide electrophoresis was run in sodium dodecyl sulphate.

sodium dodecyl sulphate) of ATPase samples previously exposed to alkaline pH (Fig. 13). An ATPase preparation consisting predominantly of band 1 was converted to band 2 by exposure to pH 10 for 3 h. If the sodium dodecyl sulphate gel pattern of the exposed protein (mainly band 2 in Fig. 2) was compared to that of untreated ATPase (mainly band 1) the most striking differences were the decrease in the molecular size of subunit α, that now overlapped with β, and the appearance of several small molecular weight components, besides γ and δ. Furthermore, δ was also probably degraded (see Fig. 13a). This provided an explanation for the irreversibility of the changes in CD by a simple return to neutral pH value.

More drastic changes occurred at pH 11 when unfolding of the ATPase started. After 1 h at pH 11 the α subunit reported by Andreu et al. [5] was completely destroyed, whereas β was still apparently unchanged (component a, relative mobility = 0.69). Subunit γ was barely detectable (component c, relative mobility = 0.78) and component d (relative mobility = 0.85) may be similar or identical to subunit δ. Several new high-mobility species appeared, perhaps arising from the degradation of subunits α, δ and γ (components b, e, f, g and other minor ones incompletely resolved). If the ATPase sample was maintained at pH 11.0 for 30 h (sample taken from the analytical ultracentrifuge run) subunit β was also destroyed and band (relative mobility = 1.00) predominated, thus confirming the results obtained by ultracentrifugation. If a protease inhibitor, benzylsulphonyl fluoride (0.5 mM), was included during the treatment at pH 11, the results were unchanged. Moreover, we did not detect any proteolytic activity at alkaline pH values in the purified ATPase, using as substrates either Azocoll or casein.

DISCUSSION

The ATPase preparation

Two main forms of the ATPase molecule were present in the solubilized, pure enzyme. These were not isoenzymes because their relative proportions could be changed by the storage at −20 °C (very slowly), by freezing and thawing, or by exposure to mildly alkaline pH (fast conversion). Of course, the final species obtained by these different methods might not be the same. The conversion of band 1 into band 2 and, to some extent, into band 3 was accompanied by an increase in the two fast moving, small molecular weight components (bands 4 and 5). These changes could also be correlated with a variation in the shape and intensity of the CD spectrum occurring in the same range of pH. The change in CD, small in magnitude, must however reflect a quite large change in conformation because the far ultraviolet CD represented the dichroism of some 3200 peptide bonds. Whether these properties observed for a soluble ATPase preparation are relevant or not to the function of the enzyme in vivo must await further work.

The acid transition of the ATPase

Circular dichroism measurements in the far ultraviolet are not likely to be a good test for dissociation of the ATPase. This is shown by the results at pH 5 where gel electrophoresis and analytical centrifugation indicated dissociation, whereas the CD spectrum was unchanged and its intensity only slightly decreased (Figs 6 and 7). The study of the acid denaturation was complicated by the onset of strong aggregation

in the transition region. This might be one important reason to account for the incomplete regain of enzymic activity in samples exposed to pH values lower than 7. The incomplete dissociation found at such low pH values as 1.5 (Table II) indicated that acid denaturation of the ATPase was a complex process proceeding in several steps. In view of this fact it is surprising the quality of the first-order kinetics plots (table I). On the other hand, the rates measured in table I probably reflected aggregation rather than or as well as unfolding. In one case the rate was measured from the increase in apparent ultraviolet absorption (due to scattering) and thus definitely reflected aggregation. Because these values correlated well with those from other experiments (pH = 3.44, 4.59) in which aggregation was not so extensive or had been reversed to a large extent, we believe that aggregation followed the unfolding of the partly dissociated protein, the latter being the rate determining process.

The alkaline unfolding transition

At pH 11 the ATPase underwent its main conformational transition on the alkaline side (Fig. 9). The reaction was fast, including presumably dissociation of subunits, unfolding of some of them and exposure of tyrosine residues that were inaccesible to hydroxyl ions at lower pH values. This fast step, which we could not study in the spectrophotometer, was followed by a slow unfolding to give a structure with a large amount of random coil. Many of the dissociated species finally observable by polyacrylamide gel electrophoresis or analytical ultracentrifugation were of a smaller molecular weight than that of the subunits of the ATPase and indeed were a product of the degradation of these subunits. The rupture of the primary structure of these polypeptide chains seems exceedingly easy. Because no conventional proteolytic activity was detected in the enzyme preparation, we are left with simple hydrolysis as the only explanation. Similar but much less extensive degradations of the subunit α could be caused by storage, freezing and thawing and exposure to pH values near 10. Neither these conditions, nor pH 11 at 14 °C are likely to lead to cleavage of peptide bonds. Therefore we would like to speculate that the subunits of the ATPase described by Andreu et al. [5] are made up of smaller peptide chains connected by linkages more labile than the peptide bond. The chemical nature of these linkages, if they exist at all, is a matter of further speculation, but ester bonds or glycosidic linkages would fulfil better the chemical requirements.

The interaction between the subunits of the ATPase molecule

Ionic strength values as high as 3 did not achieve dissociation of the ATPase molecule. This fact suggested that the interactions between the subunits of the enzyme described by Andreu et al. [5] were not of a predominantly polar character. Other results obtained in this work confirm this idea. Values of pH which should alter the charge of the protein enough to weaken polar interactions to a large extent did not bring about dissociation of the main subunits of the ATPase. The conclusion is that hydrophobic bonds must contribute predominantly to the association of these subunits. Most of the tyrosine residues of the ATPase titrated with an anomalously high pK (Fig. 10) but not in a time-dependent manner as is usual in such cases. We therefore suggest that many of these residues are in the surface of contact between the subunits.

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