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Optical Properties and Denaturation by Guanidinium Chloride and Urea of the Adenosine Triphosphatase of *Micrococcus lysodeikticus*

A COMPARISON OF FOUR MOLECULAR FORMS OF THE ENZYME

By MANUEL NIETO and JUAN A. AYALA
Sección de Bioquímica de Membranas, Centro de Investigaciones Biológicas, Velázquez 144, *Madrid-6, Spain*

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1. The fluorescence and circular dichroism of four homogeneous preparations of ATPase (adenosine triphosphatase) from *Micrococcus lysodeikticus* differing in molecular structure and enzymic properties were examined at pH 7.5 and 25°C. Emission was maximum at 325 and 335nm and the relative intensities at these wavelengths may be used to characterize the different ATPase preparations. The circular-dichroism spectra exhibited negative extrema at 208 and 220nm, and the relative value of the molar ellipticity at these wavelengths was also different for each molecular form of the enzyme. 2. The four preparations undergo two consecutive major unfolding transitions in guanidinium chloride (midpoints at 0.94 and 1.5M denaturant), with concomitant destruction of the quaternary structure of the protein. A comparatively minor alteration in the ATPase structure also occurred in 0.05–0.2M-guanidine and led to complete inactivation of the enzyme. The inactivation and the first unfolding transition were reversible by dilution of the denaturant; the transition with midpoint at 1.5M-guanidine was irreversible. 3. Similar results were obtained in urea, except that the successive transitions had midpoints at concentrations of denaturant of 0.4, 2.0 and 4.5M. Low concentrations of urea caused a noticeable activation of the enzyme activity and alterations of the electrophoretic mobility of the ATPase. 4. A model is proposed in which one of the major subunits, α, is first dissociated and unfolded reversibly by the denaturants, followed by the irreversible unfolding and dissociation of the other major subunit, β, from subunit δ and/or the components of relative mobility 1.0 in dodecyl sulphate/polyacrylamide-gel electrophoresis (ρ).

The ATPase* (bacterial F_1, BF_1, EC 3.6.1.3) of the membranes of *Micrococcus lysodeikticus* is an energy-transducing ATPase similar to those of mitochondria, chloroplasts and other bacteria (Andreu *et al.*, 1973; Penefsky, 1974; Abrams & Smith, 1974; Pedersen, 1975). It can be solubilized by very mild treatment in the absence of detergent (Muñoz *et al.*, 1969) and purified to homogeneity (Andreu *et al.*, 1973; Andreu & Muñoz, 1975).

The preparation thus obtained was the so-called form A of the enzyme. Three additional pure preparations were obtained from a closely related substrain of this micro-organism (forms B; Carreira *et al.*, 1976*a*,*b*). They differed from form A and from each other in specific activity, stimulation by trypsin and subunit apparent molecular weight and/or composition (Carreira *et al.*, 1976*a*,*b*).

In a previous paper we examined the possibility of achieving reversible denaturation of the ATPase (preparation A) by variations in the pH (Nieto *et al.*, 1975). However, we found that those conditions that led to dissociation of the ATPase molecule into the constituent subunits also led to their irreversible unfolding and chemical degradation. The observation that the enzyme could be inactivated by moderate concentrations of guanidine or urea and the activity recovered by dilution or dialysis (J. M. Andreu, unpublished work) encouraged us to study the denaturation by these compounds. In the present work we compare the fluorescence and c.d. of the enzyme preparations mentioned above and their denaturation by guanidinium chloride and urea. We show that for all the forms of the enzyme the effect of the denaturants takes place in three steps, two of which are reversible by dilution.

A preliminary account of part of this work has been presented (Nieto & Andreu, 1975).

* Abbreviations: ATPase, adenosine triphosphatase; c.d., circular dichroism.

Materials and Methods

Chemicals

Guanidinium chloride and urea were Ultrapure from Schwarz/Mann (Orangeburg, NY, U.S.A.); ATP (disodium salt) was from P-L Biochemicals

(Milwaukee, WI, U.S.A.). All other chemicals were of the best quality commercially available.

Enzyme preparations

ATPase preparations A, B_{AT}, B_A and B_I were purified as described by Andreu & Muñoz (1975) and Carreira *et al.* (1976*a*,*b*). They had the subunit composition described by the above authors, and their specific activities in μmol/min per mg of enzyme, measured by the pH-stat method (Nieto *et al.*, 1975), were the following: preparation A, 25; preparation B_I (strain PNB, inactive), 2.4; preparation B_{AT} (strain PNB, active and stimulated by trypsin), basal activity, 7, stimulated by trypsin (Carreira *et al.*, 1976*b*) to 14; preparation B_A (strain PNB, active, not stimulated by trypsin), 23. The assay conditions are described below.

Enzyme assay

ATPase activity was measured by the pH-stat method (Nieto *et al.*, 1975) or colorimetrically (Vambutas & Racker, 1965; Andreu *et al.*, 1973). Incubations were performed in 30mM-Tris/HCl buffer, pH7.5, at 37°C and had a final ATP/$CaCl_2$ concentration of 8mM. The reactions were started by adding enzyme (15 or 35 μg, depending on the activity of the preparation). The concentration of ATPase solutions was estimated by using the value of $A^{1\%}_{1cm}$= 6.9 at 276nm as previously determined (Nieto *et al.*, 1975; Carreira *et al.*, 1976*a*).

Physical measurements

U.v. absorption (Cary 16S spectrophotometer) and circular dichroism (Roussel–Jouan 185 Dicrograph II) were measured at 25±0.1°C and pH7.5 as described previously (Nieto *et al.*, 1975). The values of dichroic absorption in a light-path of 1cm, ΔA, were converted into mean residue molar ellipticity, $[\theta]$, by means of the relationship, $[\theta] = 3298\ \Delta A/c$ degree·cm^2·dmol^{-1}, where c was the mean residue concentration in mol/litre. A value of 109 was used for the mean residue weight (Nieto *et al.*, 1975).

Fluorescence was measured at 90° to the exciting beam (275nm) with a FICA 55MK II difference spectrofluorimeter (Le Mesnil, St.-Denis, France). This apparatus recorded excitation and emission spectra, corrected for lamp quantum output and detector response respectively. Settings were as follows: excitation and emission bandwidths, 7.5nm; measure gain 10; reference voltage, 540V; time-constant, 3s; scan speed, 10nm/min. The Rhodamine B cuvette was maintained at 32±0.1°C and the sample and reference cuvettes at 25±0.1°C by means of jacketed cell-holders through which water was circulated from two independent constant-temperature baths (Lauda K2R, Lauda, West Germany).

Cuvettes with light-paths of 2, 5 and 10mm were used as required to keep the absorption of the samples below 0.05. Double-glass-distilled water was used in the preparation of solutions and these, except for the protein, were identical in both the sample and reference cuvettes.

Measurements of pH (Radiometer pHM26) and analytical centrifugation (Spinco model E) by the high-speed method (Yphantis, 1964) were performed as described by Nieto *et al.* (1975).

Polyacrylamide-gel electrophoresis

Gels (5% acrylamide; 0.6cm×10cm) were prepared and electrophoresis was carried out at pH8.6 in the presence of urea as described by Andreu & Muñoz (1975). However, in our case the concentration of urea used in the polymerization of the gels and the upper buffer chamber was variable from 0 to 8M.

Electrophoresis in sodium dodecyl sulphate, pH8.6, was carried out as described by Andreu *et al.* (1973).

Denaturation by guanidinium chloride or urea

To estimate the equilibrium fraction of protein remaining native after exposure to a given concentration of denaturant, separate solutions (0.5–2.5ml) of ATPase in 30mM-Tris/HCl buffer, pH7.5, containing guanidinium chloride or urea were maintained at 25°C for 3–5h, and then one or two of the following properties measured: enzymic activity, circular dichroism or fluorescence. The minimal time to reach equilibrium had been determined in previous experiments by recording continuously the time-dependence of the circular dichroism or fluorescence emission at several concentrations of denaturant. The concentrations of enzyme used in these experiments were in the range 0.03–0.08mg/ml. In other experiments, where reversal of denaturation was tested, the enzyme concentrations in the solution of denaturant were in the range 0.2–0.5mg/ml. After these solutions had been left at 25°C for 3.5h, they were diluted 5- or 10-fold with 30mM-Tris/HCl buffer, pH7.5, and their activity, circular dichroism or emission spectra were measured. The concentration of urea and guanidinium chloride was estimated from measurements of the refractive index of the solutions (Fasman, 1963; Nozaki, 1972). Two measurements of refractive index were performed, one immediately after setting up the denaturation mixture and another after it had been left for 3–5h at 25°C. Both agreed within 2–3% in the experiments reported.

Results

Subunit composition of the ATPase preparations

A brief description of the subunit composition of the four ATPase preparations used here should facilitate the description of the results and their subsequent discussion. Subunits are all designated by Greek letters; subunit σ corresponds to subunit x of Carreira *et al.* (1976*a*,*b*). Preparation A was $\alpha_3\beta_3\gamma\delta\rho$

(Andreu *et al.*, 1973; Andreu & Muñoz, 1975). Preparations B_I and B_A were $(\alpha'+\alpha'')_3\beta_3\delta\sigma\rho$; preparation B_{AT} was $(\alpha'+\alpha'')_3\beta_3\delta\sigma\varepsilon\rho$ (Carreira *et al.*, 1976*a*,*b*). Preparations of B-type contained two kinds of subunit α, which differed in apparent molecular weight in dodecyl sulphate/polyacrylamide-gel electrophoresis. The difference between forms B_I and B_A resided in the relative proportion of these α chains. Preparation B_{AT} was identical with form B_A, except that it contained an additional subunit, ε, characterized as the intrinsic ATPase inhibitor. All the ATPase preparations had one or more components that migrated with the tracking dye in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This is called ρ.

Fluorescence and circular dichroism of the ATPase preparations

The c.d. spectra in the far-u.v. of the three molecular forms of ATPase from strain PNB of *M. lysodeikticus* (B_I, B_A and B_{AT}) are shown in Fig. 1 and compared with that of preparation A previously reported (Nieto *et al.*, 1975). They all had a similar shape, with negative extrema at 220–222 and 208–209nm. However, the magnitude of the ellipticity at these wavelengths was different, being highest for the active non-stimulated preparation (form B_A). All the preparations from strain PNB differed from form A in the value of $[\theta]_{208}$ relative to $[\theta]_{222}$ (Fig. 1).

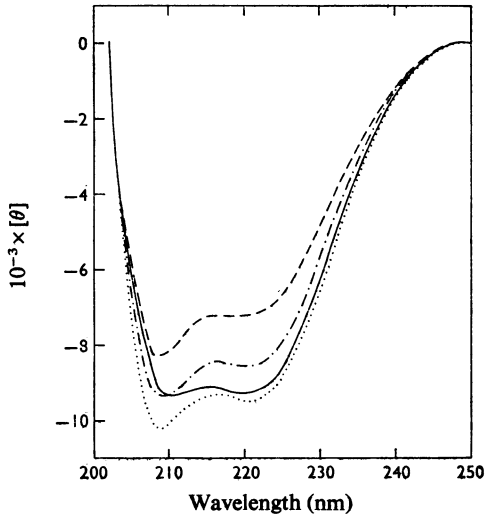

Fig. 1. *Circular dichroism of the different molecular forms of M. lysodeikticus ATPase*

Each curve was the average of at least ten spectra, each obtained in duplicate. The concentration of protein ranged from 0.03 to 0.5mg/ml and cuvettes of light-path from 0.1 to 1cm were used. All spectra were obtained in 30mM-Tris/HCl buffer, pH7.5 at 25°C. $[\theta]$ is the mean residue ellipticity (degree·cm^2·dmol^{-1}). Molecular forms: A (——); B_I (----); B_A (·····); B_{AT} (-·-·).

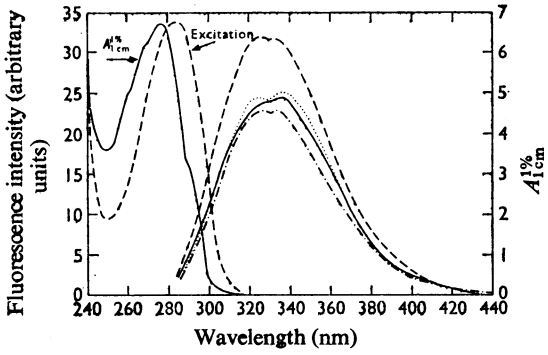

Fig. 2. *Absorption, excitation and emission spectra of the molecular forms of M. lysodeikticus ATPase*

The excitation and emission spectra were recorded in 1cm cuvettes at a protein concentration of 0.04mg/ml in 30mM-Tris/HCl buffer, pH7.5, and 25°C. Other conditions were as indicated in the Materials and Methods section. Excitation was at 275nm and the emission spectra corresponded to forms A (——), B_I (----), B_A (·····) and B_{AT} (-·-·). The excitation spectra (emission at 330nm) was that of form B_I, but its shape was identical for the other ATPase forms. For comparison, an absorption spectrum ($A^{1\%}_{1cm}$) valid for the four preparations of ATPase is also included (right-hand scale).

The c.d. at 208 or 222nm of forms A and B was linear with concentration of protein in the range 0.02–2.0mg/ml.

The corrected emission spectra of the four preparations are shown in Fig. 2. It was characteristic of tryptophan residues in globular proteins, except that two emission maxima at about 325 and 335nm were apparent. The intensity of the intrinsic fluorescence at 335nm was higher than that at 325nm in the so-called active preparations (A and B_A). In the 'inactive' (B_I) or trypsin-stimulated (B_{AT}) forms the reverse applied. Another difference was the overall fluorescence intensity, higher in form B_I than in the other three ATPase forms.

The shape of the corrected excitation spectra was similar in the four preparations, so only that of form B_I is shown (Fig. 2). It had a maximum at about 283nm and showed a displacement to longer wavelengths in comparison with the absorption spectrum of the protein ($\lambda_{max.}$ 276nm; Fig. 2).

The intensity of emission at 330nm was in all cases linear with ATPase concentration in the range from 0.005 to at least 0.4mg/ml, provided that the light-path was chosen to obtain a maximum absorption of 0.07. Higher values were not tested. After correction

for inner filter effect the emission spectra were not altered by the presence of 0.06mM-ATP, 10mM-$CaCl_2$, 10mM-$MgCl_2$ or combinations of these.

The fluorescence of all the preparations of ATPase decreased during illumination of the samples with light from the spectrofluorimeter lamp. This was probably due to photolysis and was measured at pH7.5 and 25°C by exciting at 275nm and recording the fluorescence at 330nm as a function of time. Preparation B_A lost 37% of its fluorescence after being exposed to light of 275nm for a period of 4.5h. The rate constants of photolysis, obtained from first-order plots of $\ln[(F-F_{final})/(F_{initial}-F_{final})]$ against time, where F was the fluorescence intensity, were $7.4\times10^{-3}min^{-1}$ for preparation B_I, and $8.1\times10^{-3}min^{-1}$ for preparation B_A. Photolytic decomposition was faster and more extensive in old enzyme preparations. Thus a sample of B_A stored for 2 years at −20°C lost 60% of its fluorescence in 3.5h with a rate constant of $1.7\times10^{-2}min^{-1}$.

The loss of enzymic activity during photolysis was faster than that of emission intensity: the rate constant for the inactivation of form B_A was $1.5\times10^{-2}min^{-1}$. The loss of activity reached apparently a maximum after 4.5h exposure to light, when 56% of the initial activity remained. To minimize photolysis, the exposure to light of the samples was restricted to the short period required to perform measurements. At all other times the cell compartment was isolated from the light-source by means of shutters.

Denaturation by guanidinium chloride

Denaturation by guanidinium chloride was studied at 25°C and pH7.5 by observing the changes produced in far-u.v. circular dichroism, fluorescence and enzymic activity, by a given concentration of denaturant, as described in the Materials and Methods section. The variation of the first three properties as a function of the concentration of guanidinium chloride is shown in Fig. 3. The dichroic absorption at 222nm, ΔA, decreased regularly as the concentration of guanidinium chloride increased from 0 to 0.5M, then showed a major change at concentrations of denaturant between 0.7 and 1M and a second less sharp transition between 1M- and 2M-guanidinium chloride. All the ATPase preparations (A, B_{AT}, B_A and B_I) showed the same behaviour, except for the overall magnitude of the variation in ΔA (Fig. 3*a*). When these curves were normalized and the fraction of native protein was plotted against the concentration of guanidinium chloride, all the points were on the same curve, within experimental error. The midpoints of the two transitions occurred at approx. 0.94M and 1.5M denaturant respectively.

The shape of the c.d. spectrum in guanidinium chloride (Fig. 4) was typical of proteins in this denaturant and showed a pronounced increase in disordered secondary structure. These changes in

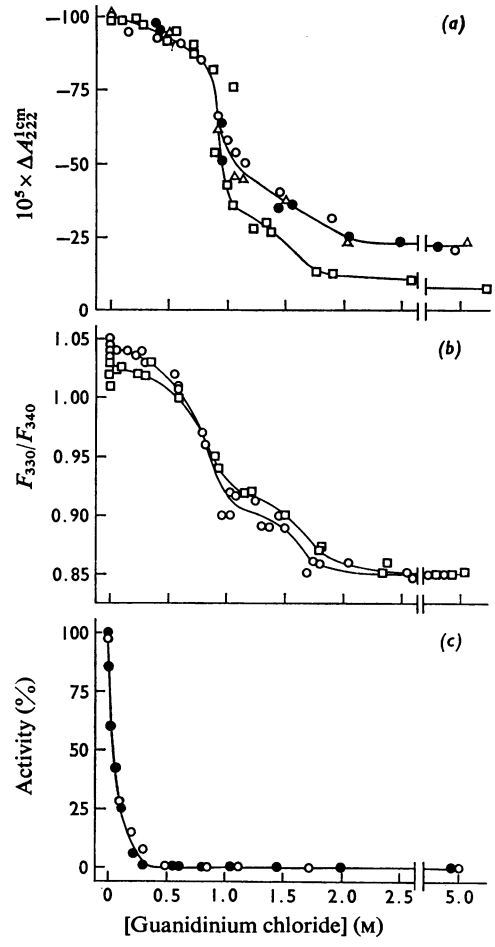

Fig. 3. *Effect of guanidinium chloride on the circular dichroism (a), fluorescence (b) and enzymic activity (c) of M. lysodeikticus ATPase*

The experimental points correspond to preparations A (□), B_I (○), B_{AT} (△) and B_A (●). Preincubation to equilibrium (3–5h) with the denaturant was performed at 25°C and pH7.5. In (*a*), ΔA is the measured value of the c.d. as difference in absorption for left- and right-hand circularly polarized light in a 1cm light-path. The value of the dichroism in the absence of guanidine has been given the arbitrary value of −100 for all the ATPase preparations. In (*b*), F_{330}/F_{340} represents the emission of the protein at 330nm relative to that at 340nm when fluorescence was excited at 275nm. Only forms B_I and A are represented. The shape of the curves for forms B_A and B_{AT} was similar to that of the curve for preparation A. (*c*) shows the activity of a protein preparation exposed to a given concentration of denaturant for 3.5h at 25°C and pH7.5 relative to that of a control maintained under identical conditions in the absence of guanidinium chloride. For further experimental details, see the Materials and Methods section.

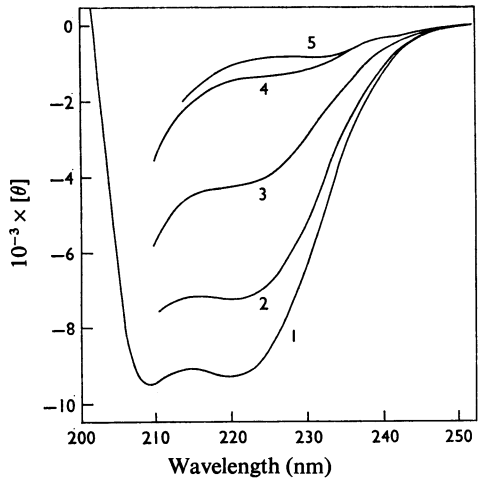

Fig. 4. *Effect of guanidinium chloride on the far-u.v. circular dichroism of ATPase (preparation A)*
The spectra were recorded after maintaining the protein for 4h at 25°C and pH7.5 (30mM-Tris/HCl buffer) in the following concns. of guanidinium chloride: curve 1, 0M; curve 2, 0.75M; curve 3, 0.96M; curve 4, 1.93M; curve 5, 5.6M-guanidinium chloride containing 0.2mM-dithiothreitol. [θ] is the mean residue ellipticity (degree·cm^2·dmol^{-1}).

the c.d. spectra were reversible by dilution of the guanidinium chloride provided that a concentration of about 1.1M was not exceeded. The reversion was too fast to be followed with a conventional recorder and was not influenced by ATP (8mM), $CaCl_2$ (8mM), $MgCl_2$ (8mM) or combinations of these. It was also insensitive to β-mercaptoethanol (3mM) or dithiothreitol (3mM).

The denaturation of the protein could also be followed by the decrease in fluorescence intensity at a chosen wavelength, for example 330nm or, more conveniently, by the shift in the $\lambda_{max.}$ of emission occurring on unfolding of the peptide chains. This shift, measured by means of the ratio of fluorescence intensities at 330nm and 340nm, is shown in Fig. 3(*b*) as a function of concentration of guanidinium chloride. If other ratios were chosen, for instance F_{325}/F_{340}, F_{335}/F_{340} or F_{330}/F_{345}, the results were essentially the same. All the enzyme preparations behaved similarly and only forms B_1 and A are shown. From 0 to 0.35M-guanidinium chloride the fluorescence intensity at 330nm decreased by only about 2%, and the position of the maximum emission remained unchanged. At higher concentrations two unfolding transitions were apparent, with midpoints near 0.9M and 1.6M denaturant respectively. These transitions were probably identical with those observed in c.d. at similar concentrations of guanidine.

The shape of the emission spectrum of the ATPase at two chosen concentrations of guanidinium chloride is shown in Fig. 5 for preparation B_1. The results were the same for the other forms of the enzyme. During the transition with midpoint near 0.9M, the $\lambda_{max.}$ was shifted to 340nm, and the spectrum began to develop a shoulder near 305nm (Fig. 5). In the following transition (midpoint 1.6M) this shoulder developed into a second maximum of emission ($\lambda_{max.}$ 303–305nm), whereas the main peak was further shifted to the red ($\lambda_{max.}$ about 347nm). The maximum of emission at 303nm was not present in tryptophan or lysozyme under similar conditions; it could be quenched by making the solutions 0.3–1.0M in KI together with the maximum at 347nm; and finally, in old or photolysed ATPase samples it became 1.3 times as intense as the maximum at the longer wavelength.

The corrected excitation spectra at emission wavelengths of 305 and 345nm are also shown in Fig. 5 together with the absorption spectra in 6M-guanidinium chloride. The $\lambda_{max.}$ of absorption was near 275nm, and those of excitation occurred at 280nm ($\lambda_{emission}$ 305nm) and 285nm ($\lambda_{emission}$ 345nm).

The changes caused to the fluorescence of the enzyme by guanidinium chloride could be reversed by dilution provided that a denaturant concentration of about 1.0M was not exceeded. The reversion at 25°C and pH7.5, as observed in both fluorescence and c.d., was very fast, and its kinetics could not be followed in a conventional dichrograph or fluorimeter. The changes induced by exposure to concentrations of guanidinium chloride higher than 1.1M could not be reversed by dilution or dialysis, at either 25° or 0°C in the presence or the absence of dithiothreitol (3mM).

The four ATPase preparations were inactivated by low concentrations of guanidinium chloride (Fig. 3*a*). In 0.05M denaturant 50% of the activity was lost, and at a concentration of 0.4M the enzyme was totally inactive. The presence of 8mM-ATP or 8mM-$CaCl_2$ did not protect the activity to any detectable extent. As observed for the optical properties, the enzymic activity was totally recovered by dilution, provided that the enzyme had not been exposed to guanidinium chloride concentrations higher than 1.1M. Beyond this concentration no activity could be recovered by dilution or dialysis, whether in the presence or the absence of ATP (8mM), $MgCl_2$ (8mM) or $CaCl_2$ (8mM).

The state of association of the subunits of the ATPase at several concentrations of guanidinium chloride was examined by using analytical ultracentrifugation as described by Yphantis (1964). The plots of log$J$ against r^2 for preparation B_1 are shown in Fig. 6. Generally, two or three straight-line segments were observed in the presence of the denaturant. The slopes of these segments were calculated by

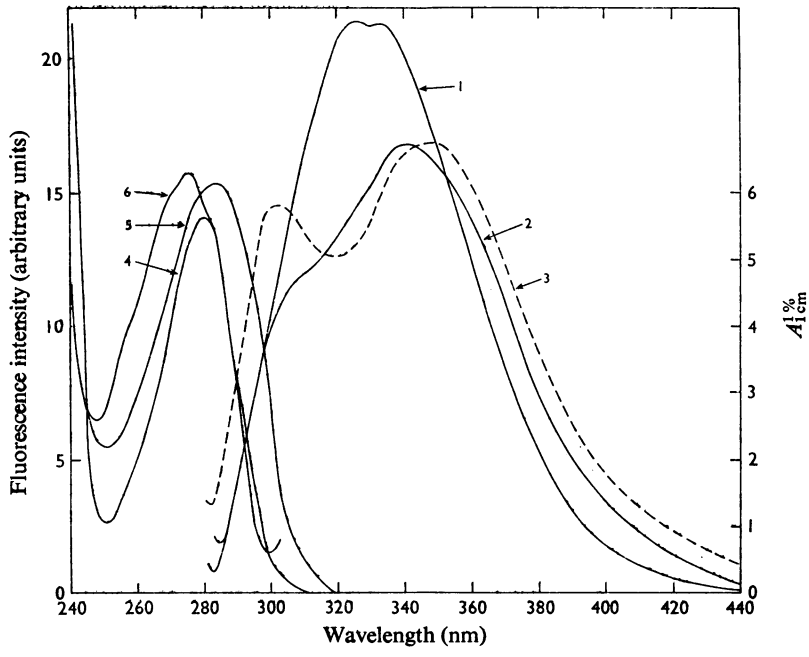

Fig. 5. *Effect of guanidinium chloride on the excitation and emission spectra of M. lysodeikticus ATPase*
Emission spectra (excitation at 275nm): curve 1, 0; curve 2, 1.21M-guanidinium chloride; curve 3, 4.7M-guanidinium chloride. Excitation spectra were in 4.7M-guanidinium chloride: curve 4, emission at 305nm; curve 5, emission at 345nm. The absorption spectrum of the enzyme in 6M-guanidinium chloride (curve 6, right-hand scale) has also been included for comparison purposes. All the spectra are for preparation B_1, 0.069mg/ml in a light-path of 0.5cm. They were recorded after exposing the protein to the denaturant for a period of 3h at 25°C and pH7.5 (30mM-Tris/HCl buffer). The same results were obtained with the other ATPase preparations.

using a least-squares program, and the derived values of the weight-average molecular weight are shown in Table 1. These values give us only an indication about the overall state of aggregation of the subunits, but too many species were present for a rigorous analysis to be carried out. Partial dissociation occurred at 0.59M-guanidinium chloride, and became fairly extensive in 0.89M and 1.0M denaturant. However, at the end of the first transition observed in c.d. and fluorescence, subunit dissociation was not yet completed, as suggested by the experiment in 1.5M-guanidinium chloride (Table 1). The presence of a single straight line in 6M-guanidine may be due to the choice of the baseline for fringe measurement. Indeed, at this concentration of denaturant the meniscus was never totally cleared, as reported by Farron (1970) for chloroplast ATPase.

Kinetics of denaturation by guanidinium chloride

The rate of peptide-chain unfolding and exposure of tryptophan residues to the solvent was measured at 25°C and pH7.5 by recording the circular dichroism at 222nm or the intensity of emission at 330nm with time. First-order plots of $\ln[(P-P_f)/(P_i-P_f)]$ (P, property measured; i, initial value at $t=0$; f, final value at $t=\infty$) against time were constructed, and straight lines adjusted by means of a least-squares program. A single straight line was obtained in the range of guanidinium chloride concentrations from 0.9 to 1.3M. From 1.3 to 2.2M denaturant, two straight lines of fairly different slope were observed, and again a single one in 2.56M-guanidinium chloride. Finally, at a concentration of 3M or higher the unfolding was so fast that it could not be followed with a conventional recorder. The apparent rate constants, i.e. the negative of the slopes of these straight lines, are shown in Table 2. The range of guanidinium chloride concentrations that caused the presence of one or two straight lines in the first-order plots corresponded closely to that causing one or both unfolding transitions. So it appears that the two major denaturation steps occurred consecutively in time, i.e. a given concentration of denaturant capable of inducing both unfolding transitions caused first the transition with midpoint at 0.94M-guanidinium

chloride and then that with midpoint at 1.5M-guanidinium chloride.

The three ATPase preparations examined were unfolded by guanidinium chloride at a similar rate (Table 2).

Denaturation of M. lysodeikticus ATPase by urea

The effect of urea on the fluorescence and enzymic activity of ATPase is shown in Fig. 7. As with guanidinium chloride the dependence of F_{330}/F_{345} on the concentration of denaturant followed a curve with an intermediate plateau. The midpoints of the two transitions thus defined were 2.0M- and 4.5M-urea respectively (Fig. 7*a*). The maximum emission, centred near 330nm in the native protein, was shifted to 340nm in 2.6M-urea, and to 303 and 347nm in 6.0M-urea. Therefore the behaviour of the fluorescence spectrum of ATPase in urea was very similar to that in guanidinium chloride, except for the range of concentrations involved.

On the other hand, the effect on the enzymic activity of both denaturants was partially different. Fig. 7(*b*) shows that low concentrations of urea stimulated the ATPase activity of the protein up to about 175% of that in the absence of the compound. At higher concentrations of urea the enzyme lost the stimulation, and then was totally inactivated by 2M-urea. The midpoint of the inactivation was near 1.6M-urea.

The changes in both fluorescence and activity were

Table 1. *Weight-average molecular weight of M. lysodeikticus ATPase in solutions of guanidinium chloride*
The protein solution (0.5–0.7mg/ml) in 50mM-Tris/HCl buffer, pH7.5, contained the concentrations of denaturant indicated in the Table. The temperature of the rotor was 13–14°C and a double-sector 12mm cell was used in all the experiments. The measured density of the solutions containing guanidinium chloride was in agreement with the values reported by Kawahara & Tanford (1966). A value of 0.74ml/g was used for the partial specific volume of the protein in both buffer and guanidinium chloride solutions (Andreu *et al.*, 1973). The ATPase preparation B_1 was used in all the experiments.

Concn. of guanidinium chloride (M)	Rotor speed (rev./min)	Weight-average mol.wt.
0.00	10000	368000
0.59	10000	212000
		283000
		346000
0.89	20000	36300
		53000
		90000
1.00	26000	21000
		29000
		51000
1.50	34000	17000
		22300
		32700
6.0	36000	37000

Table 2. *Apparent rate constant of unfolding of M. lysodeikticus ATPase by guanidinium chloride*
The experiments were carried out at 25°C. Denaturation was initiated by adding guanidinium chloride (8M, in 30mM-Tris/HCl buffer, pH7.5) to a solution of ATPase in 30mM-Tris/HCl buffer, pH7.5, or ATPase to a buffered solution of the denaturant. The decay in c.d. at 222nm or fluorescence intensity at 330nm (excited at 275nm) was recorded continuously until no further charges occurred (value of the property at $t = \infty$). Fluorescence measurements were carried out intermittently to minimize photolysis. The value of $\ln[(P-P_\infty)/(P_i-P_\infty)]$ (P, property measured; P_i, its value at zero time, and P_∞ the equilibrium value after the termination of the transition at $t = \infty$) was plotted against time and k values calculated as $k = -$slope.

ATPase preparation	Method of observation	Guanidinium chloride concn. (M)	k_1 (s^{-1})	k_2 (s^{-1})
A	C.d.	0.96	5.0×10^{-4}	
		1.01	4.6×10^{-4}	
		1.15	2.4×10^{-3}	
		1.3	2.5×10^{-3}	
		1.93	—	2.5×10^{-3}
B_I	Fluorescence	0.88	4.9×10^{-4}	
		2.02	—	5.7×10^{-3}
B_A	C.d.	1.03	4.7×10^{-4}	
		1.63	1.14×10^{-2}	1.5×10^{-3}
		2.17	1.92×10^{-2}	7.1×10^{-3}
		2.56		6.2×10^{-2}
		3.02		$>10^{-1}$

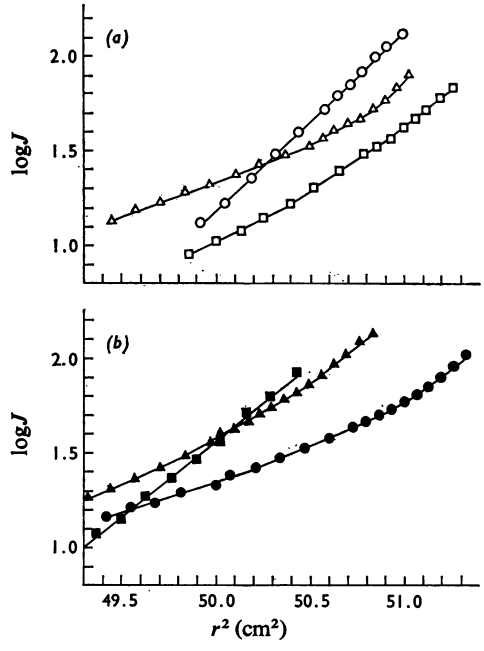

Fig. 6. *Sedimentation equilibrium of ATPase (B_1) in guanidinium chloride*
The concentrations of guanidinium chloride and rotor speeds were the following: ○, control without guanidine, 10000rev./min; □, 0.59M, 10000rev./min; △, 0.89M, 20000rev./min; ●, 1.0M, 26000rev./min; ▲, 1.5M, 34000rev./min; ■, 6.0M, 36000rev./min. All the denaturant solutions were in 50mM-Tris/HCl buffer, pH7.5. Fringe patterns were recorded on Kodak metallographic plates and the measurements of fringe displacement (J) with the radial distance (r) made by using a Nikon model 6c profile projector. Two light and two dark fringes were measured in each experiment and the results averaged. For other conditions see Table 1.

reversible in samples exposed to a maximum concentration of urea of about 2.5M. When the enzyme was treated with concentrations of denaturant higher than that, neither dilution nor dialysis, in the presence or the absence of ATP (8mM), $CaCl_2$ (8mM) or $MgCl_2$ (8mM), could achieve reversion.

The products of the treatment of ATPase with a given concentration of urea at 25°C and pH7.5 during 4h were subjected to polyacrylamide-gel electrophoresis in that concentration of urea at pH8.5. Densitometric scans of the gels after staining with Coomassie Blue are shown in Fig. 8. In the range of urea concentrations where the enzyme activity was stimulated (0–0.5M-urea), a single band was present whose mobility relative to that of the tracking dye decreased from 0.75 to 0.64. Several bands could be observed in 1.0M-urea, but again,

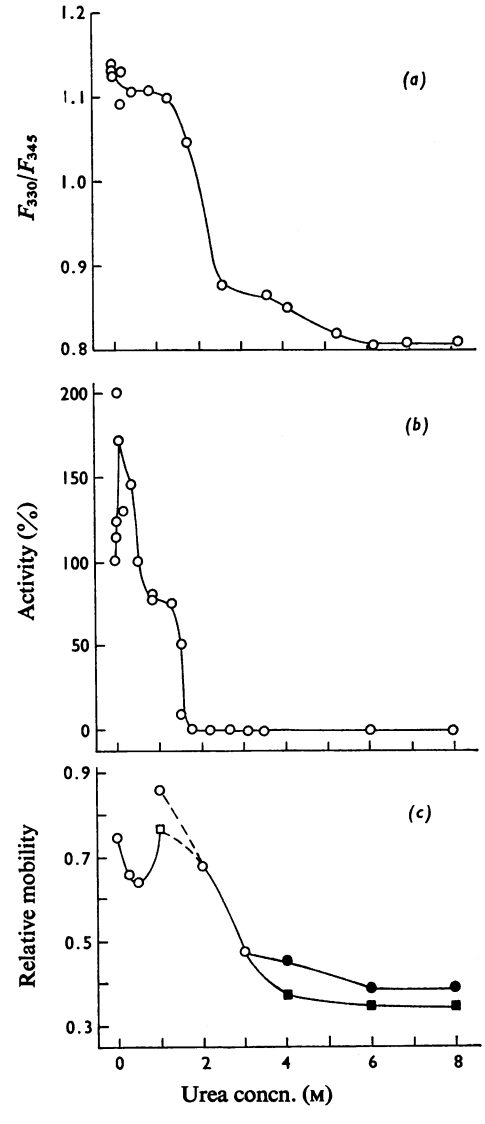

Fig. 7. *Effect of urea on the fluorescence (a), activity (b) and electrophoretic mobility (c) of M. lysodeikticus ATPase*
The experimental points are for preparation B_A. The results obtained with the other enzyme forms were very similar. The variables measured on the ordinates of (*a*) and (*b*) and the experimental conditions were as in Fig. 3. In (*c*) we show the values of the relative mobility of the main protein band (○) as a function of the concentration of urea at which the denaturation and subsequent gel electrophoresis was carried out. When two symbols appear for a given concentration of denaturant (○, □), two main species were observed after staining the gels. In 4M- (or higher) urea the two main subunits were separated (●, subunit α; ■, subunit β). For further details see Fig. 8.

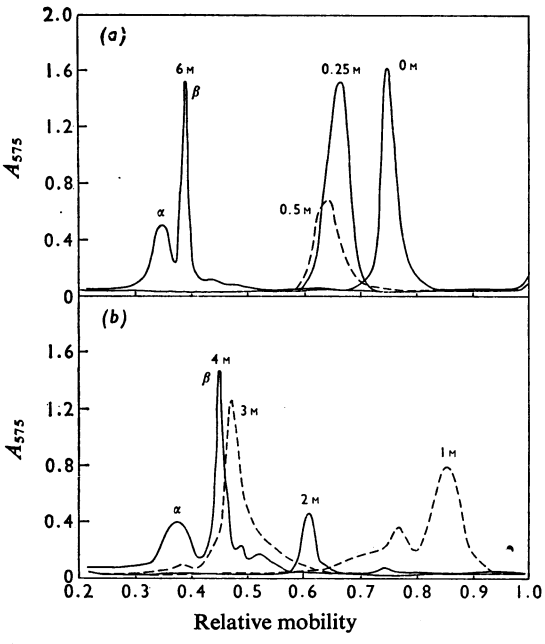

Fig. 8. *Polyacrylamide-gel electrophoresis of ATPase in various concentrations of urea*

The protein samples (34 μg) were pre-exposed for 4h at 25°C and pH7.5 (30mM-Tris/HCl buffer) to the same concentration of urea as that present in the gel and electrode upper chamber. After addition of a drop of 50% (v/v) glycerol and tracking dye the samples were applied to the gels (5% acrylamide) and these were run in jacketed tubes maintained at 20–25°C. Migration was towards the cathode. The gels were stained with Coomassie Brilliant Blue R250 (Fairbanks *et al.*, 1971) or Coomassie Blue G250 (Diezel *et al.*, 1972). Scanning was performed with a model 2400 Gilford spectrophotometer equipped with a 2410-S linear transport system. Form B_A was used in these experiments, but similar results were obtained for the other ATPase preparations. Under the scanning conditions used subunits α' and α'' were not resolved from each other. The concentrations of urea were the following: (*a*) control without urea (0.0M); 0.25M and 6M (——); 0.5M (----). (*b*) 2M and 4M (——); 1M and 3M (----). The concentrations are indicated in the Figure. The bands corresponding to the major subunits (α and β) have also been identified in 4M and 6M urea.

only one in 2.0M denaturant; the staining capacity of the protein, which began to diminish in 0.5M, reached a minimum in 2.0M-urea, but was regained at higher concentrations of the compound. Then the relative mobility decreased (3.0M-urea), and finally dissociation products that could be identified with the major subunits of ATPase (α and β; Andreu & Muñoz, 1975) appeared in 4.0M-urea. From 4M- to 8M-urea the overall electrophoretic pattern remained unchanged. The relative mobility of the main bands, as judged by staining intensity, is plotted in Fig. 7(*c*) as a function of the concentration of urea. At those concentrations of urea where major changes in fluorescence or activity took place, these were accompanied by alterations of the relative mobility.

Discussion

Optical properties of the ATPase preparations

The differences previously observed between the ATPase preparations as regards molecular properties and enzymic activity (Nieto *et al.*, 1975; Carreira *et al.*, 1976*a*,*b*) were also reflected in the fluorescence and circular dichroism (Figs. 1 and 2). These optical properties seem to be convenient to characterize the preparation and study their possible interconversion.

The secondary structure of form A, estimated from its c.d. spectrum (Greenfield & Fasman, 1969), contained about 20% α-helix (Nieto *et al.*, 1975); by using the same method of estimation and values of c.d. in the same range of wavelength, form B_I was found to contain 16% α-helix. These values compare fairly well with 22% α-helix in ox heart mitochondrial ATPase (Warshaw *et al.*, 1968), 20% in spinach chloroplast CF_1 (Farron, 1970), 20% in *Bacillus stearothermophilus* (Hachimori *et al.*, 1970) and 27% α-helix in the ATPase from a very thermophilic bacterium (Yoshida *et al.*, 1975).

The emission spectra of the four ATPase preparations from *M. lysodeikticus* were characteristic of tryptophan residues inside globular proteins. However, the presence of two maxima of emission in the native protein, at 325 and 335nm, indicated that two families of tryptophan residues existed that differed in the polarity of their environments. Because the critical distance for energy transfer between tryptophan residues is about 1.6nm (Chen *et al.*, 1970, and references therein) it is simplest to assume that each tryptophan family occurred in a different type of ATPase subunit. Similarly, the separation of tyrosine and tryptophan emissions observed after complete dissociation and unfolding of ATPase in concentrated guanidinium chloride or urea (Fig. 5) suggests the existence of yet another type of subunit that does not contain tryptophan. Considering that other ATPases, such as those from spinach chloroplast (Farron, 1970) or *Rhodospirillum rubrum* chromatophore (Berzborn *et al.*, 1975), did not contain any tryptophan at all, and that the presence of this residue is doubtful in the mitochondrial enzyme (Penefsky, 1974; Pedersen, 1975), it is not surprising to find that some of the subunits of *M. lysodeikticus* ATPase lack this amino acid. The presence of two maxima in the emission spectrum of heat-denatured ATPase from *Bacillus stearothermophilus* (Hachimori & Nosoh, 1973) can be inter-

preted similarly. According to these authors, ATPase contained 1.6% of tryptophan residues. The spectra of the enzymes from spinach chloroplast and *R. rubrum* chromatophore had a single maximum of emission at 303–310nm (Lien & Racker, 1971; Berzborn *et al.*, 1975).

Denaturation by guanidinium chloride and urea

The four ATPase preparations, differing in enzymic activity and molecular and optical properties, nevertheless showed identical behaviour in guanidinium chloride and urea. Therefore their overall structures and the type and strength of the non-covalent bonds that maintain them must be very similar.

When we consider together the effect of guanidinium chloride or urea on the enzymic, optical and molecular properties of the protein, at least three transitions can be defined in either denaturant. The transitions observed in urea were probably very similar to those occurring in guanidinium chloride, except that the latter was more effective than urea in bringing them about.

The first transition had a midpoint at about 0.05M-guanidinium chloride or 0.4M-urea as deduced from Figs. 3(*c*) and 4(*b*). It manifested itself mainly by sharp variations in the enzymic activity and changes in the electrophoretic mobility of the protein (Figs. 3 and 7). The inhibition of the ATPase activity by high ionic strength has already been described by Nieto *et al.* (1975) and this might account for the effect of guanidinium chloride. On the other hand, the fluorescence of the enzyme was virtually unaffected and the circular dichroism only showed a small decrease. Therefore we may imagine the transition as a limited change in the conformation of one or more of the ATPase subunits; this would cause an initial decrease and then an increase in the surface negative charge of the protein as indicated by the variations in its electrophoretic mobility in the presence of urea. The decrease in the negative charge of the protein surface would facilitate the approach and binding of the negatively charged substrate, thus explaining the initial enhancement of the enzymic activity in urea.

The second transition occurred at 0.94M-guanidinium chloride or 2M-urea, and was characterized by an extensive unfolding of the peptide backbone (c.d. spectra, Figs. 3 and 4), and exposure to the solvent of a large proportion of the tryptophan side chains (emission spectra, Figs. 3 and 5). The rate constant k_1 (Table 2) probably corresponded to this transition. Only partial subunit dissociation took place concomitantly with these changes in conformation (Table 1). At the end of this transition in urea the protein reached a minimum of staining capacity in polyacrylamide gels. So the observation of a single band in the gels in 2M-urea (Fig. 8) might be misleading; other species unable to be stained may be present. In fact, if a sample of protein was treated with 2M-urea for 3h at 25°C and then subjected to gel electrophoresis in sodium dodecyl sulphate, pH8.6, one of the major subunits (α) could not be detected by staining the gels with Coomassie Blue R250 or G250. We have no explanation for these findings, but they constitute a serious technical hindrance in the molecular characterization of the denaturation products.

The last transition (midpoint at 1.6M-guanidinium chloride or 4.5M-urea) achieved complete dissociation and unfolding of the enzyme and the separated subunits recovered their ability to be stained normally with Coomassie Blue (Fig. 8). The apparent rate constant k_2 (Table 2) probably corresponded to this transition. In contrast with those discussed previously, this transition could not be reversed by dilution or dialysis. Assuming that the two large conformational changes observed by means of fluorescence and c.d. represent the successive unfolding of the major subunits α and β, then it seems that one of them had to conserve most of its native structure for reversion to be possible. The loss of staining capacity of subunit α in 2M-urea suggested that this was the subunit that was unfolded first. Therefore the last irreversible transition would correspond to the unfolding of subunit β. In the last denaturation step, the separation of the emission of tyrosine from that of tryptophan also occurred. This phenomenon was interpreted as the dissociation of a subunit containing tryptophan from another that lacked this amino acid residue. The isolated major subunits, α and β, both contained tryptophan (J. M. Andreu & E. Muñoz, personal communication). Hence the subunit(s) lacking this residue should be δ and/or ρ, i.e. the remaining subunits common to the four ATPase preparations studied here. So in the denaturation model proposed, subunits δ and/or ρ must remain associated to subunit β until the last transition. The overall process could be formulated as follows:

$$\alpha_3\beta_3\delta\rho \rightleftharpoons (\alpha_3\beta_3\delta\rho)\ddagger \rightleftharpoons 3\alpha+\beta_3\delta\rho \rightarrow 3\alpha+3\beta+\delta+\rho$$

where $(\alpha_3\beta_3\delta\rho)\ddagger$ denotes the product of the first transition, a quasi-native ATPase differing from the native protein mainly in the surface charge.

This sequence does not seem very consistent with the symmetrical hexagonal model proposed for the native protein (Catterall & Pedersen, 1974; Kozlov & Mikelsaar, 1974), but would be in good agreement with the 'stalk' model (Kozlov & Mikelsaar, 1974).

Systematic studies on the denaturation of energy-transducing ATPases from other sources have not been reported. However, Vogel & Steinhart (1976) have isolated two fractions from the cold-inactivated ATPase from *Escherichia coli* that contained subunits $\alpha\gamma\varepsilon$ and β respectively. Reassociation to give an

active protein occurred by mixing the two fractions in the presence of ATP/Mg^{2+} (5mM). These results suggest a denaturation model similar to ours, except that in *E. coli*, subunit β might be that initially dissociated from the ATPase complex.

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