Thermal Denaturation of *Micrococcus lysodeikticus* Adenosine Triphosphatase

INFLUENCE OF TEMPERATURE ON THE CIRCULAR DICHROISM, FLUORESCENCE AND ENZYMIC ACTIVITY OF THE PROTEIN

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The soluble ATPase (adenosine triphosphatase) from *Micrococcus lysodeikticus* underwent a major unfolding transition when solutions of the enzyme at pH 7.5 were heated. The midpoint occurred at 46°C when monitored by changes in enzymic activity and intrinsic fluorescence, and at 49°C when monitored by circular dichroism. The products of thermal denaturation retained much secondary structure, and no evidence of subunit dissociation was detected after cooling at 20°C. The thermal transition was irreversible, and thiol groups were not involved in the irreversibility. The presence of ATP, adenylyl imidodiphosphate, CaCl₂ or higher concentrations of ATPase conferred stability against thermal denaturation, but did not prevent the irreversibility once denaturation had taken place. In the presence of guanidinium chloride, thermal denaturation occurred at lower temperatures. The midpoints of the transition were 45°C in 0.25 M-, 38°C in 0.5 M- and 30°C in 0.75 M-denaturant. In the highest concentration of guanidinium chloride a similar unfolding transition induced by cooling was observed. Its midpoint was 9°C, and the temperature of maximum stability of the protein was 20°C. The discontinuities occurring in the Arrhenius plots of the activity of this enzyme had no counterpart in variations in the far-u.v. circular dichroism or intrinsic fluorescence of the protein at the same temperature.

The energy-transducing ATPases (EC 3.6.1.3) as solubilized from the membranes of mitochondria, chloroplasts and bacteria are very complex multi-subunit proteins whose structure and function are still poorly understood. Many of the problems posed in this respect could be successfully approached if it were possible to dissociate the enzyme reversibly into its constituent subunits. Our attempts to carry out dissociation and reconstitution of the ATPase by a method similar to that used for other multimeric enzymes (Cook & Koshland, 1969) were not successful. Therefore we started a systematic study of the environmental conditions that lead to reversible and irreversible dissociation and unfolding of the *Micrococcus lysodeikticus* ATPase.

In previous papers we examined the dissociation and unfolding of the ATPase at 25°C by variations in the pH and ionic strength (Nieto *et al.*, 1975) or in the presence of guanidinium chloride and urea (Nieto & Ayala, 1977). In the present work we describe the effect of variations in temperature on the conformation of the enzyme, in the presence or absence of guanidinium chloride.

Abbreviations used: ATPase, adenosine triphosphatase; p[NH]ppA, 5'-adenylyl imidodiphosphate.

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Energy-transducing ATPases are generally cold-labile. Although the enzyme from *M. lysodeikticus* is reasonably stable to cold, it was hoped to detect conformational transitions induced by low temperatures, similar to those observed for other proteins (Brandts, 1969).

A brief description of the subunit composition of the ATPase preparations used here should facilitate the comparison with energy-transducing ATPases from other sources as well as help in the description of the results.

Subunits are all designated by greek letters. Its molecular weight and stoichiometry were studied by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Preparations B₁ and B_A contained ($\alpha' + \alpha''$)₃ $\beta_3\delta$. In form B₁ both α' and α'' chains were in about equal amounts and had a mol.wt. of 50000. In form B_A the content of α' (mol.wt. 60000) generally exceeded that of α'' (mol.wt. 55000). The molecular weight of β was 50000 and that of δ , 30000, in both preparations (Carreira *et al.*, 1976).

Molecular forms B₁ and B_A always contained another two components whose stoichiometry, as judged by intensity of staining with Coomassie Blue, was variable. These were components σ (mol.wt. 97000) previously called X by Carreira *et al.* (1976),

and component(s) ρ . The latter run with the tracking dye in polyacrylamide-gel electrophoresis. The status of these bands as true subunits of ATPase is still under consideration.

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Materials and Methods

Chemicals

ATP (disodium salt) was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.), and Sephadex G-200 and Blue Dextran were from Pharmacia (Uppsala, Sweden). p[NH]ppA was a product of ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Guanidinium chloride (ultra-pure) was purchased from Schwartz-Mann (Orangeburg, NY, U.S.A.). Lysozyme and phosphorylase A were products of Boehringer (Mannheim, West Germany); pepsin, bovine serum albumin, aldolase, egg albumin and tryptophan were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade, generally from Merck (Darmstadt, West Germany).

ATPase preparation

Micrococcus lysodeikticus N.C.T.C. 2665 sub-strain PNB was grown and its membrane ATPase solubilized and purified to greater than 95% purity as described previously (Carreira *et al.*, 1976). Because of its greater stability the molecular form of the enzyme generally used was that designated B₁ (Carreira *et al.*, 1976). It had a specific activity of 2.5 $\mu\text{mol}/\text{min}$ per mg when assayed at 37°C and pH 7.5 by using the pH-stat method, as described below. Critical experiments were also performed with the ATPase preparation designated B_A (Carreira *et al.*, 1976; Nieto & Ayala, 1977). The results obtained with both enzyme preparations were essentially the same.

ATPase assay

The protons liberated during the hydrolysis of ATP were measured by using a Radiometer pH-stat assembly as described by Nieto *et al.* (1975). The reaction mixture was maintained at $37 \pm 0.02^\circ\text{C}$ with the help of a jacketed reaction vessel through which water was circulated from a constant-temperature bath (Lauda K2RD, Lauda, West Germany). The electrode, a combined semi-micro instrument from Radiometer (Copenhagen, Denmark), was standardized in the pH range 4–9, as described by Bates (1973). The reaction mixtures (volume 1.5 ml) had the following final concentrations: ATP, 8 mM;

CaCl₂, 8 mM; KCl, 30 mM. The reaction was started by the addition of 30–60 μg of ATPase and performed under N₂ at pH 7.5.

ATPase concentration

This was determined from the scattering-corrected absorption at 276 nm and the value of $A_{1\text{cm}}^{1\%} = 6.9$ previously determined (Carreira *et al.*, 1976). The values thus obtained were in very good agreement with colorimetric determinations by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard.

Other techniques

Polyacrylamide-gel electrophoresis at pH 8.5, in the presence or absence of sodium dodecyl sulphate, was performed as described by Andreu *et al.* (1973) in gel rods (0.6 cm $\times$ 12 cm). The gels were stained with Coomassie Brilliant Blue R250 (Fairbanks *et al.*, 1971) or G250 (Diezel *et al.*, 1972) and scanned at 575 and 550 nm respectively, with a Gilford 2400 spectrophotometer equipped with a model 2410 S linear-transport system.

U.V. absorption, c.d. and intrinsic fluorescence were measured as described previously (Nieto *et al.*, 1975; Nieto & Ayala, 1977).

During optical measurements the temperature inside the cuvettes was monitored continuously by means of a thermocouple connected to a digital thermometer (YEW 2509; Yokogawa Electrical Works, Tokyo, Japan).

Denaturation experiments

All the solutions were made in deionized double-glass-distilled water. Stock solutions of ATPase (5–10 mg/ml) in 0.1 M-Tris/HCl buffer, pH 7.5, were maintained at -20°C until required.

To monitor the changes in the conformation of the enzyme we used the change in c.d. at 222 nm and the shift in the position of the emission maximum (Nieto & Ayala, 1977). The latter was measured as the ratio of the fluorescence intensity at 330 nm to that at 340 nm (excitation at 275 nm). The equilibrium experiments were of two general types. (a) To a solution (2 ml) of KCl (30 mM), in a cuvette of 10 mm light-path, ATPase was added (10 or 20 μl , by Carlsberg micropipette) to a final concentration of 50 μg of protein/ml and 1 mM-Tris/HCl buffer, pH 7.5. If guanidinium chloride was used, then KCl was replaced by 30 mM-Tris/HCl buffer, pH 7.5. The cuvette was stoppered, placed in the thermostatically controlled cell holder and the temperature varied in steps of 2 or 5°C. At each temperature the c.d. or the fluorescence were recorded with time until they

reached their final values. (b) Separate protein solutions similar to those described in (a) were placed in constant-temperature baths and maintained at the desired temperature until equilibrium had been reached. The time required (15 min–10 h) had been previously determined in kinetic experiments. The solution was then transferred to a cuvette thermostatically maintained at the same temperature as that of the preincubation, and the c.d. or fluorescence were measured.

This method was also used to determine the fraction of ATPase remaining active after exposure to a given temperature until equilibrium. In this case the final ATPase concentration in the preincubation mixtures was either 56 $\mu\text{g/ml}$ or 1 mg/ml. The enzymic activity was always assayed at 37°C.

The kinetics of unfolding were studied by using reaction mixtures as described in (a). They were thermostatically maintained in the Dicrograph cuvette (Roussel-Jouan 185 Dicrograph II, Paris, France) at the required temperature, and recording of c.d. at 222 nm was initiated immediately after adding ATPase; it was continued until no further change in the c.d. took place.

The concentration of guanidinium chloride was estimated from measurements of the refractive index of the solutions (Nozaki, 1972). Determinations were made at the beginning and at the end of the experiment; they were in agreement within 2–3% in the experiments reported. The values of pH were also monitored at the beginning and the end of each experiment.

To test the reversibility of the transitions, the enzyme solutions, denatured as described above, were cooled or warmed in steps of 2, 5 or 10°C at a rate of 0.2°C/min. After the desired temperature had been obtained, the c.d. of the protein solution was recorded until a stable value was reached. This usually took 5–20 min. At 20°C the c.d. of the protein solution was measured during at least 2 h.

Gel chromatography

Samples of ATPase (50–70 μg in 200–300 μl of buffer), native or after thermal denaturation, were applied to a column (0.9 cm $\times$ 28 cm) of Sephadex G-200. The column was packed in and eluted with 30 mM-Tris/HCl buffer, pH 7.5, and calibrated by using tryptophan, Blue Dextran and the proteins listed under 'Chemicals' above; their molecular weights were as follows: phosphorylase *a*, 490 000; aldolase, 160 000; bovine serum albumin, 66 000; egg albumin, 44 000; pepsin, 35 000; lysozyme, 14 600. The absorption of the effluent at 276 and 206 nm was monitored with a Uvicord III instrument, model 2089 (LKB, Bromma, Sweden). The column was eluted at 0.1–0.2 ml/min with the aid of a peristaltic pump (Sage Instruments, Cambridge, MA, U.S.A.).

Results and Discussion

Influence of temperature on the fluorescence and c.d. of ATPase at pH 7.5

The equilibrium c.d. and fluorescence of soluble ATPase solutions from *M. lysodeikticus* as described in the Materials and Methods section were examined at several temperatures in the absence of guanidinium chloride; a selection of the spectra obtained is shown in Fig. 1. The c.d. of the native protein in the far u.v. had two negative maxima at about 208 and 222 nm respectively (Nieto & Ayala, 1977), the wavelength and intensity of which varied as the enzyme was heated from 20 to 70°C. The dichroic absorption at 222 nm decreased noticeably, whereas that maximum initially at 208 nm was shifted to shorter wavelengths. The major changes in c.d. occurred in the temperature range 45–55°C, where the spectra had shapes and intensities intermediate between those shown at 20 and 58°C. An example is the c.d. spectrum at 49°C shown in Fig. 1. At all temperatures higher than 55°C the spectra were virtually the same as that shown for 58°C.

After heating had produced its maximum effect on the c.d. spectrum, the protein still retained a considerable amount of folded structure, as indicated by the comparison with its c.d. in 6 M-guanidinium chloride at 25°C (Fig. 1), where the ATPase was completely dissociated and unfolded (Nieto & Ayala,

Fig. 1. Effect of temperature on the far-u.v. circular dichroism of ATPase

Curve 1, native protein at 20°C in 1 mM-Tris/HCl buffer, pH 7.5, containing 30 mM-KCl; curve 2, after a similar enzyme solution had been kept for 8 h at 49°C; curve 3, after 1 h at 58°C; curve 4, ATPase totally unfolded by 6 M-guanidinium chloride at pH 7.5 and 25°C. The enzyme concentration in all the experiments was 56 $\mu\text{g/ml}$. $[\theta]$ is the mean residue ellipticity.

Table 1. Variation of the secondary structure of *M. lysodeikticus* ATPase during thermal denaturation

The secondary-structure composition of the ATPase samples was estimated from their c.d. spectra in the range 208–240 nm by using the method of Greenfield & Fasman (1969). The fit between calculated and experimental spectra was good between 220 and 240 nm and poor at shorter wavelengths.

Temperature (°C)	Percentage of amino acid residues		
	α -Helix	β -Sheet	Without long-range order
20	15	28	57
40	15	26	59
49	11	27	62
60	9	26	65
Cooled from 60 to 20	9	27	64

1977). However, the disorganization caused by high temperatures could not be reversed by cooling. The presence of 3 mM-dithiothreitol during the heating and subsequent cooling did not modify these results. Therefore oxidation of thiol groups did not seem to be involved in the irreversibility.

We have described (Ayala *et al.*, 1976, 1977) the presence of discontinuities in the Arrhenius plots that describe the dependence on temperature of the enzymic activity of this ATPase. The discontinuities occurred near 30°C and could only be attributed to the protein itself (Ayala *et al.*, 1976). However, we found no parallel changes in the far-u.v. c.d. or the intrinsic fluorescence of the protein in the temperature range 25–35°C. So the molecular events leading to the discontinuity in the Arrhenius plot were not conformational changes at all, or were not large enough to be reflected in the magnitude or shape of the c.d. spectrum. Also they did not affect the polarity of the environment of the tryptophan residues of the enzyme.

The c.d. of the protein was examined from 20 to 2°C in 30 mM-Tris/HCl buffer, pH 7.5 and from 20 to –8°C in the same buffer containing 50% (v/v) glycerol. Only a slight increase in the mean residue ellipticity was observed with decreasing temperature. Glycerol itself at that concentration did not affect the c.d. of the activity of ATPase.

The secondary structure of the protein was analysed by using the standard c.d. curves and methods of Greenfield & Fasman (1969) or those of Saxena & Wetlaufer (1971). The best fit between calculated and experimental c.d. curves was obtained by using the standard conformations of poly-L-lysine (Greenfield & Fasman, 1969). The fit was rather poor in the region of 208–212 nm, and the reported conformational composition (Table 1) must be treated with caution. The main event in the thermal transition seemed to be an increase in the content of the structure without long-range order at the expense of the α -helix. The content of β -structure remained essentially unaltered during thermal unfolding.

Fig. 2. Effect of temperature on the intrinsic fluorescence of ATPase

Excitation was at 275 nm. Curve 1, native protein at 20°C in 1 mM-Tris/HCl buffer, pH 7.5, containing 30 mM-KCl; curve 2, after a similar protein solution had been kept at 49°C for 8 h; curve 3, after 1 h at 60°C; curve 4 (···), protein solution exposed to 60°C for 1 h and then cooled back to 20°C; curve 5 (---), ATPase completely unfolded by 5.0M-guanidinium chloride at 25°C and pH 7.5. The enzyme concentration in all the experiments was 50 µg/ml.

The fluorescence emission spectra of the protein at several temperatures (excitation at 275 nm), obtained under the same conditions as those of c.d., is shown in Fig. 2. The native protein had two emission maxima, at 325 nm and 335 nm (Nieto & Ayala, 1977). When the enzyme was heated, a single broad maximum of emission was observed, shifted to longer wavelengths with respect to the native enzyme. At 55°C the emission maximum was at 338–339 nm. A shoulder at 305–310 nm, already present at 40°C, did not develop into another independent maximum at higher temperatures (Fig. 2). We have included in Fig. 2 the emission spectrum of the protein in 5M-guanidinium chloride, where the

protein was dissociated and its peptide chains were completely unfolded (Nieto & Ayala, 1977). A comparison with the emission spectrum at 60°C shows that thermal denaturation did not achieve complete unfolding of the ATPase chains, as indicated above by the c.d. spectra.

Thermal transition at pH7.5

The variation in c.d. and fluorescence brought about by increasing the temperature of a solution of the enzyme in 30 mM-KCl/1 mM-Tris/HCl buffer, pH7.5, is shown in Fig. 3. The transition observed in the c.d. at 222nm had a midpoint at 49°C (Fig. 3a), whereas the shift in emission maximum, measured by the ratio of the fluorescent intensity at 330nm to that at 340nm (F_{330}/F_{340}), had half-maximum value near 46°C (Fig. 3b). Both changes were irreversible in either the presence or the absence of 3 mM-dithiothreitol.

The enzymic activity at 37°C measured after prolonged exposure (see under 'Kinetics of denaturation') to temperatures in the range from 20 to 70°C is shown in Fig. 3(c). The irreversible loss of activity had a midpoint at about 46°C and correlated well with the changes in the fluorescent emission.

Loss of enzymic activity with temperature was also examined at a protein concentration of 1.0 mg/ml (see the Materials and Methods section). Under these conditions the midpoint of inactivation was higher (49–50°C). The simplest interpretation for this increase in stability with enzyme concentration is that inactivation depended on the dissociation, governed by the law of mass action, of an ATPase fragment. This fragment may be nucleotide. A protein sample containing 1.3 mol of externally added ^3H -labelled nucleotide/mol of enzyme that was exposed to 100°C for 10 min lost 27% of this bound nucleotide on subsequent gel filtration, whereas a similar sample kept for the same period at 20°C lost only 16% of its bound nucleotide (J. A. Ayala & M. Nieto, unpublished work).

Kinetics of denaturation

The decrease in c.d. at 222nm with time when solutions of the enzyme (30 mM-KCl/1 mM-Tris/HCl buffer, pH7.5) were exposed to temperatures in the

Fig. 3. Thermal denaturation of ATPase monitored by changes in (a) c.d. at 222nm, (b) fluorescence and (c) loss of enzymic activity

●, Experimental points obtained by continuous heating to equilibrium at increasing values of temperature for the same enzyme solution (experiments of type *a* in the Materials and Methods section). ○, Experiments in which separate protein solutions were heated from 20°C to the desired temperature and left at that temperature until equilibrium was reached (15 min to 9–10 h; see Fig. 4). This type of experiment was described in the Materials and Methods section as type *b*. ▲, Values of c.d. at 222nm or F_{330}/F_{340} obtained by cooling down the protein solutions at a rate of 0.2°C/min and leaving them at the temperature indicated by the experimental point for a time from 15 min to 2 h. Slower or faster rates of cooling or prolonged (up to 15 h) exposure to 20°C in the presence of 3 mM-dithiothreitol or 2 mM-ATP did not change the irreversibility of the process. The concentration of protein used was 50–56 $\mu\text{g/ml}$ and the solutions were in 1 mM-Tris/HCl buffer, pH7.5, containing 30 mM-KCl. ΔA_{222} is the measured value of c.d. at 222nm as the difference in absorption between left- and right-hand circularly polarized light in a 1 cm light-path. F_{330}/F_{340} is the emission of the protein at 330nm relative to that at 340nm when fluorescence was excited at 275nm. The enzyme activity of ATPase solutions exposed to equilibrium to a given temperature was assayed as 37°C.

Fig. 4. Kinetics of thermal unfolding of ATPase
The experiments were performed on protein solutions similar to those of Fig. 3 as indicated in the Materials and Methods section. The c.d. at 222 nm ($\Delta A_{222}^{1\text{cm}}$; ●) in a light-path of 1 cm was continuously recorded until it did not undergo any further change with time. The variations of enzymic activity (○) with time at a given temperature were determined by using separate protein solutions immersed in the same constant-temperature bath. The activity was always assayed at 37°C and was expressed as a percentage of that of the native enzyme kept at 20°C for the same period. (a) Change in c.d. with time at 47°C (left-hand scale); change in activity with time at 45°C (right-hand scale); (b) change in c.d. at 49°C; enzymic activity at 50°C; (c) change in c.d. at 51°C; (d) change in c.d. at 53°C; (e) changes in c.d. and activity at 55°C.

transition region is shown in Fig. 4 and compared with the loss of enzymic activity under similar conditions. At least two different kinetic processes, con-

secutive in time, could be observed in the temperature range 45–51°C. The second and slower of these appeared to be responsible for the irreversible inactivation of the enzyme (Figs. 3a, 3b and 3c). At temperatures higher than 51°C, only one process could be detected. First-order plots of

$$\ln [(\Delta A - \Delta A_t)/(\Delta A_i - \Delta A_t)]$$

(ΔA , c.d. at 222 nm; ΔA_i initial value at $t = 0$; ΔA_t , final value at $t = \infty$) against time were constructed, and straight-line segments were fitted visually. Two straight-line segments were obtained in the temperature range 45–51°C and a single straight line at higher temperatures. The negative of the slopes of these straight-line segments was termed the 'apparent rate constant' of the process. The values of these rate constants at several temperatures are shown in Table 2, which also shows the values for the cold transition in 0.75 M-guanidinium chloride, where two straight lines were also obtained in the first-order plot.

Thermal transitions in the presence of guanidinium chloride

The dependence on temperature of c.d. at 222 nm and F_{330}/F_{340} for ATPase solutions at pH 7.5 containing guanidinium chloride is shown in Fig. 5. The general behaviour of the optical properties of the protein in guanidinium chloride concentrations from 0.025 to 1.0 M in the range of temperatures 20–50°C was similar to that in the absence of the compound, except that the transition occurred at lower temperatures as the concentration of denaturant increased. As observed by c.d., the transitions without denaturant and in 0.25 M-guanidinium chloride were virtually identical. Therefore in Fig. 5(a) the filled triangles (▲) are the experimental points in 0.25 M denaturant, whereas the continuous line without symbols that goes beyond the experimental points is that of Fig. 3(a), obtained in the absence of guanidinium chloride. The midpoints of the transitions were as follows: in 0.25 M-guanidinium chloride, 45°C observed by fluorescence and 49°C by c.d.; 0.5 M, 38°C by both methods; and 0.75 M, 30°C. The protein was denatured at all temperatures in 1.0 M- and 1.5 M-denaturant (Nieto & Ayala, 1977; Fig. 5).

The thermal unfolding of the peptide chains in the presence of concentrations of guanidinium chloride up to 1.0 M was only partial as observed in the absence of denaturant. The c.d. spectrum of the protein at 60°C in 1.0 M-guanidinium chloride was quite similar in shape and magnitude to that recorded at the same temperature in the absence of the compound.

The emission spectra of the heat-denatured protein pointed to the same conclusion. The emission maxima occurred at 339 nm (0.25 M-guanidinium chloride at

Table 2. *Apparent rate constants for the thermal unfolding and inactivation of M. lysodeikticus ATPase*

The experiments were carried out as described in the Materials and Methods section. Guanidinium chloride was excluded except in the study of the kinetics of the 'cold transition' (last line of Table, k values at 1°C). Values of $\ln [(\Delta A - \Delta A_t)/(\Delta A_i - \Delta A_t)]$ (ΔA , c.d. at 222nm; ΔA_i , initial value at $t = 0$; ΔA_t , final value at $t = \infty$) were plotted against time and k values calculated as $k = -\text{slope}$. At each temperature at least two experiments were performed and the k values averaged. In the temperature range 45–51°C and at 1°C, two straight-line segments were observed for the c.d. data. The rate constants obtained from the slopes of these segments have been designated k_1 and k_2 ; K is the rate constant of inactivation of the enzyme.

Temperature (°C)	Circular-dichroism data		Enzymic-activity data K (min ⁻¹)
	k_1 (min ⁻¹)	k_2 (min ⁻¹)	
45	—	—	4.7×10^{-3}
47	4.3×10^{-2}	5.8×10^{-3}	—
49	10.3×10^{-2}	5.34×10^{-3}	—
50	—	—	8.3×10^{-3}
51	$\geq 7.5 \times 10^{-2}$	1.04×10^{-2}	—
53	—	3.9×10^{-2}	—
55	—	4.3×10^{-2}	3.0×10^{-2}
58	—	9.9×10^{-2}	—
63	—	6.9×10^{-1}	—
1	5.8×10^{-2}	7.14×10^{-3}	—

60°C), 340nm (0.5M-denaturant, 55°C), 341nm (0.75M, 40°C); and 342nm (1.0M, 35°C). These values show that the tryptophan side chains were never completely exposed to the solvent. A shoulder was always observed near 303–305nm, but never an independent maximum as in 5M-denaturant (Fig. 2).

Another observation was the tendency of the emission maxima to revert to shorter wavelengths at the end of the transition. This was reflected as an increase in the value of F_{330}/F_{340} as the temperature was raised beyond that at which the transition occurred (Fig. 5), and was particularly obvious at guanidinium chloride concentrations from 0.5 to 1.0M. Thus in 1.0M-denaturant the emission maximum occurred at 342nm at 35°C, but returned to 339–340nm at 55°C. This phenomenon reflected a limited re-burial of tryptophan side chains with increasing temperature beyond the transition point.

A more profound change took place in 1.5M-guanidinium chloride. The c.d. and fluorescence spectra of the protein at this concentration of the compound and 45°C were virtually identical with those in 6M-guanidinium chloride, where the protein was totally dissociated and its peptide chains unfolded (Nieto & Ayala, 1977).

Cold denaturation

A conformational transition induced by cooling could not be observed in the absence of guanidinium chloride or at low concentrations of the compound. However, it was easily induced by cooling in the presence of 0.75M-denaturant (Figs. 5a and 5b). The midpoint of the transition was 9°C and the temperature of maximum stability of the protein was 19–21°C,

in agreement with all the empirical evidence obtained in the handling of the enzyme.

The transition was irreversible. Neither the native c.d. nor the fluorescence of the enzyme could be recovered by slowly re-warming the solution to 20°C (Fig. 4) or by leaving it at 20°C overnight in the presence of ATP.

The magnitude of the c.d. at 222nm and the F_{330}/F_{340} ratio, and the shape of the c.d. and fluorescence spectra at the end of the cold transition, were very similar to those observed for the heat-denatured enzyme at 45°C in the same concentration of guanidinium chloride. This suggests that the final products of both transitions may be very similar.

Protection from thermal inactivation by ATP, p[NH]-ppA and Ca^{2+}

This was examined in the absence of guanidinium chloride at pH7.5. The stability of the ATPase activity to increases in temperature in 30mM-KCl was compared with that of similar protein solutions containing ATP (10mM), CaCl_2 (10mM) and p[NH]ppA (1.5mM). p[NH]ppA was a non-hydrolysed competitive inhibitor of the enzyme ($K_i = 34 \mu\text{M}$; M. Nieto, unpublished work). The concentrations of ATP and inhibitor used were saturating.

Both nucleotides had a strong protective effect on the enzyme, increasing the midpoint of the inactivation from 46°C to 53°C (ATP) and 56°C (p[NH]-ppA). A more limited protective effect was shown by CaCl_2 ; the midpoint of the inactivation in its presence was 49°C. None of the compounds modified the irreversibility of the thermal inactivation once it had taken place.

Fig. 5. Thermal transitions in the c.d. (a) and intrinsic fluorescence (b) of ATPase in the presence of guanidinium chloride

The protein solutions (50–56 µg/ml) were in 30 mM-Tris/HCl buffer, pH 7.5. The c.d. at 222 nm of the native protein at 20°C has been given the arbitrary value of $10^{-5} \times -100$ and other experimental values are corrected accordingly. The curve showing the transition in the absence of denaturant is represented as a solid line without experimental points. This curve fitted reasonably well the experimental points for the c.d. in 0.25 M-guanidinium chloride (▲). Other concentrations of denaturant were as follows: △, 0.5 M; ○, 0.75 M; □, 0.99 M; ■, 1.5 M (in b only). Filled circles (●) were obtained by warming up the product of the cold transition in 0.75 M-guanidinium chloride. The meaning of ΔA and F_{330}/F_{340} is the same as in Fig. 3.

Characterization of the products of thermal denaturation in the absence of guanidinium chloride

The products of thermal denaturation in the absence of guanidinium chloride at temperatures from 45 to 51°C were subjected to polyacrylamide-gel electrophoresis in 7 and 5% (w/v) gels. The results for 7% gels are shown in Fig. 6. The band corresponding to native protein (R_m 0.27) decreased a little

in relative mobility as the temperature increased. At the same time its staining capacity was strongly diminished and a new band developed that had a mobility relative to that of the tracking dye of 0.02. No clear evidence of subunit dissociation could be found. A similar result was observed for 5% gels. In this case the relative mobility of the native ATPase (0.55) was virtually unchanged on denaturation and a second band of R_m 0.07 developed. This band of low relative mobility could be due to aggregation and/or a change in the surface charge of the enzyme.

If the denaturation products were electrophoresed in the presence of sodium dodecyl sulphate, a pattern similar to that of the native protein was obtained, except for the total or almost-total loss of staining capacity of subunit α . No equivalent staining capacity was found elsewhere in the gel.

Solutions of ATPase treated similarly were chromatographed on a column of Sephadex G-200 as indicated in the Materials and Methods section. Native ATPase had K_D 0.03, corresponding to a mol.wt. of about 360000. Thermal denaturation did not change this value. Products with a molecular weight greater than that of native ATPase were therefore unlikely to be present. Dissociation into products of a molecular weight similar to that of the subunits, or aggregates of these of a molecular weight lower than that of ATPase, were not observed.

Dissociation and unfolding of the subunits of ATPase

The results presented here emphasize the importance of hydrophobic interactions in the maintenance and evolution of the structure of the *M. lysodeikticus* ATPase. A similar conclusion was reached from the studies of denaturation by pH (Nieto *et al.*, 1975) and urea and guanidinium chloride (Nieto & Ayala, 1977). This made denaturation at high temperatures unsuitable to achieve total dissociation and unfolding of the protein.

These hydrophobic interactions must be stronger in the ATPase from *M. lysodeikticus* than they were in the ATPases from mitochondria or chloroplasts, because the bacterial enzyme was considerably more cold-stable than those from organelles (Penefsky & Warner, 1965; Lien & Racker, 1971). In fact the preparation B₁ of the bacterial enzyme used here could be frozen and thawed at pH 7.5 repeatedly without loss of activity or change in its optical properties (Carreira *et al.*, 1976). Also, the unfolding of this ATPase at low temperature required the addition of guanidinium chloride.

Nieto & Ayala (1977) have described a two-step denaturation of this enzyme at 25°C and pH 7.5 caused by guanidinium chloride or urea. The thermal denaturation of *M. lysodeikticus* ATPase was only partial and the optical properties of the denatured protein were very similar to those of the first step of

the unfolding caused by 1 M-guanidinium chloride at 25°C. However, the second step, brought about by increasing the denaturant concentration to 1.5 M at 25°C, could not be achieved by increasing or decreasing the temperature of a solution of enzyme in 1.0 M-guanidinium chloride. The importance of hydrophobic bonds and their increasing strength with increasing temperature (Némethy & Scheraga, 1962) favoured the observed limited refolding of the denaturation products rather than further unfolding of the peptide chains. The thermal transitions were

irreversible, whereas the first step of the unfolding caused by guanidinium chloride or urea at 25°C was irreversible. Thus although the products of both transitions have similar optical properties, they are not identical. Indeed, 1 M-guanidinium chloride caused partial dissociation of the ATPase at 25°C, whereas no evidence of dissociation was found for the thermally denatured protein in the absence of guanidinium chloride.

During our studies on the denaturation of *M. lysodeikticus* ATPase (Nieto *et al.*, 1975; Nieto & Ayala, 1977) we observed repeatedly that dissociation of the protein into subunits did not occur under milder conditions of pH, urea or neutral salt concentration than those required for the unfolding of subunits. In the present paper we show that unfolding of some of the subunits may occur without dissociation. This points to a very compact quaternary structure maintained mainly by hydrophobic interactions as strong or stronger than those that maintain the tertiary structure of the subunits.

Another repeated observation was that unfolding of the subunits when it did occur was generally irreversible. At mild alkaline pH values, unfolding occurred together with covalent degradation of the subunits (Nieto *et al.*, 1975). The degradation was not due to proteinases and has now also been observed at moderately acidic pH values (pH 2–3; M. Nieto, unpublished work). The explanation put forward was that some of the covalent linkages in the ATPase subunits were ester bonds or sugar linkages (Nieto *et al.*, 1975). The presence of sugars in the major subunits of *M. lysodeikticus* ATPase has been reported by Andreu *et al.* (1976). If this is confirmed the chances of achieving completely reversible unfolding of the ATPase subunits seem small. Indeed, the majority of the sugar residues of glycoproteins are very likely added after the polypeptide sequence

Fig. 6. Thermal denaturation of ATPase followed by polyacrylamide-gel electrophoresis

The protein samples (15 μ g) in 30 mM-Tris/HCl buffer, pH 7.5 (300 μ l), were incubated at the temperatures indicated for the period necessary to reach a constant value of c.d. at 222 nm (see Fig. 4). After cooling to room temperature (23–26°C) and adding a drop of 50% (v/v) glycerol and Bromophenol Blue the samples were applied to gels (7%, w/v, acrylamide) run in jacketed tubes maintained at 20°C. Migration was toward the cathode. The gels were stained with Coomassie Blue R250 (Fairbanks *et al.*, 1971) and scanned at 575 nm with a model 2400 Gilford spectrophotometer equipped with a 2410-S linear-transport system. The mobilities of the protein bands relative to that of the tracking dye were as follows: native ATPase (20–40°C), 0.27; after thermal denaturation (60°C), 0.02.

is completed (Hughes, 1976) and presumably folded to its most stable conformation. Therefore, the final physiologically active tertiary structure of glycoprotein needs no longer to be the most stable thermodynamically. The probability of its being recovered after unfolding, therefore, must in general be very low.

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