Molecular Properties of Random Coil and Refolded Forms of α and β Subunits of an Energy Transducing ATPase from Bacterial Membranes†

José Manuel Andreu and Emilio Muñoz*

ABSTRACT: The molecular weights of random coil subunits of a coupling factor (ATPase from *Micrococcus lysodeikticus*) were estimated by 8 M urea gel electrophoresis (α, 54 000 ± 10 000; β, 53 000 ± 10 000), gel chromatography in 6 M guanidine hydrochloride (α, 57 500 ± 2200; β, 53 200 ± 2200), and high-speed sedimentation equilibrium. Straight application of this technique gave much lower molecular weights (<25 000) of α and β subunits. This was due to the presence of a minor constituent (≤5%) of low molecular weight resulting from an autodegradative process of the subunits and was corrected by the application of a two-species plot [Roark, D. E., & Yphantis, D. A. (1970) *Ann. N.Y. Acad. Sci. 164*, 245–278] to obtain 55 400 ± 8600 for α and 53 600 ± 7700 for β. Molecular weights of α, 62 200 ± 2500, and β, 58 000 ± 2400, were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The significance and limitation of the molecular weight method for the determination of subunit stoichiometry are discussed. On this ground, a subunit stoichiometry is suggested for *M. lysodeikticus* ATPase (molecular weight >345 000) that includes three copies of each of the major subunits (α and β). In the presence of 6 M guanidine hydrochloride, the isolated subunits and ATPase gave separate fluorescence of exposed tyrosine and tryptophan residues, as well as circular dichroism spectra characteristic of random coil polypeptides. Both α and β subunits refolded in neutral aqueous buffer, where they had fluorescence emission maxima at 328 and 340 nm, respectively. Native ATPase had maxima at 325 and 336 nm (excitation was at 275 nm). Ellipticity values ($[\theta]_{208}$) of refolded α and β and native ATPase were −4350, −8900, and −8300 deg·cm²·dmol⁻¹, respectively. The refolded β subunit aggregated in aqueous buffer at neutral pH. The aggregation was reversed by mild detergents and increased by high ionic strength. Refolded α subunit showed a moderate aggregation that was reversed by increasing the ionic strength. Charge shift electrophoresis [Helenius, A., & Simons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 529–532] demonstrated that refolded α and β subunits interacted with mild detergents, an indication of amphiphilic character.

The adenosine triphosphatases or F_1[1] coupling factors represent the terminal enzyme in the energy path to ATP in all energy-transducing membrane systems, where they catalyze the formation of ATP at the expense of proton gradients. All the F_1 factors so far studied are oligomeric proteins which act as peripheral membrane components. The enzymes purified from mitochondria, chloroplasts, and bacterial plasma membranes show a great number of similar properties (Senior, 1973; Penefsky, 1974; Abrams & Smith, 1974; Pedersen, 1975; Nelson, 1976) but differ in the number and nature of their minor subunits. Purified bacterial ATPases or BF_1 factors from Gram-positive cocci and bacilli (Andreu et al., 1973; Mirsky & Barlow, 1972) possess the simplest subunit pattern amongst the F_1-ATPases. BF_1 from *M. lysodeikticus* is less cold-labile than the F_1 from mitochondria and *Escherichia coli* and possesses a ten times lower ATPase activity. It is made up of only 3 defined subunits (Andreu et al., 1973), unlike F_1 from mitochondria, chloroplasts, *Escherichia coli*, and the thermophile bacterium PS_3 which consists of 4–5 subunits (Pedersen, 1975; Vogel & Steinhart, 1976; Yoshida et al., 1977a,b). There is as well a controversy about the number of copies that make up the different oligomeric F_1 factors (Pedersen, 1975; Baird & Hammes, 1977).

Subunits from mitochondrial and chloroplast ATPases have been isolated (Knowles & Penefsky, 1972; Nelson et al., 1973) but no attempts to carry out renaturation have been reported. More recently adenosine triphosphatase activities were reconstituted from complexes containing different subunit proportions of *E. coli* ATPase (Vogel & Steinhart, 1976) and from purified individual subunits of the ATPase of the thermophilic bacterium PS_3 (Yoshida et al., 1977a,b). In the former study, partial dissociation of *E. coli* ATPase was achieved under conditions which probably did not involve polypeptide unfolding, whereas, in the latter, the conditions were strong enough to produce protein denaturation, though this effect was not demonstrated on the thermostable, highly resistant BF_1 from PS_3. However, none of these reports characterized physicochemically the isolated subunits and reconstituted complexes so as to provide a molecular explanation of the reconstitution experiments. A distinction should be made between simple reassociation experiments from isolated, native-like subunits and experiments that involve refolding from random coil polypeptides aimed to study their reassembly.

In our case, the subunits of *M. lysodeikticus* BF_1 could not be dissociated unless fully denaturing conditions were employed. The two major subunits (α and β) were purified by preparative polyacrylamide gel electrophoresis in 8 M urea (Andreu et al., 1976). We have carefully estimated the molecular weights of denatured α and β subunits and have studied the conditions required for their refolding into native-like structures and the properties of the refolded polypeptides.

Materials and Methods

Reagents. Acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine, all of electrophoretic grade, were obtained from Eastman Kodak. Guanidine hy-

† From the Sección Bioquímica de Membranas, Instituto de Immunología y Biología Microbiana, Velázquez 144, Madrid 6, Spain. *Received November 2, 1978.* Supported by a grant from Fondo Nacional para el Desarrollo de la Investigación (E.M.) and by a Fundación Juan March fellowship (J.M.A.).

[1] Abbreviations used: F_1, the water-soluble portion of the coupling factor or adenosine triphosphatase complex; BF_1, bacterial F_1; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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drochloride and urea, both UltraPure, were from Schwarz/Mann. Sepharose 6B and blue dextran were purchased from Pharmacia; tryptophan, tyrosine, dithiothreitol, mercaptoethanol, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), Ultrol grade, were from Calbiochem. Sodium dodecyl sulfate, specially pure, from BDH Chemicals Ltd., Triton X-100 and cetylmethylammonium bromide from Sigma Chemical Co., and sodium deoxycholate from Fisher Scientific Co. were the detergents used. The following proteins were used as molecular weight standards under denaturing conditions: lysozyme (mol wt 14 500, Calbiochem); soybean trypsin inhibitor (22 700, Boehringer); trypsin (23 000, Calbiochem); aldolase (40 000, Sigma); egg albumin (43 500, Sigma); catalase (57 500, Calbiochem); bovine serum albumin (67 000, Sigma, delipidated); urease (83 000, Boehringer); and phosphorylase *a* (92 500, Boehringer). *M. lysodeikticus* depleted membranes, i.e., membranes freed from peripheral proteins, were used as a source of intrinsic membrane proteins (Muñoz et al., 1970; Estrugo et al., 1972). Coomassie R250 was from Schwarz/Mann and Coomassie G250 from Serva Feinbiochemica. ATP disodium salt was from P-L Biochemicals. Imidazole (grade I, crystalline), agarose for use in electrophoresis, and quinine sulfate were obtained from Sigma Chemical Co., and epiandrosterone was from Roussel-Jouan.

Purification of Micrococcus lysodeikticus ATPase and Its α and β Subunits. The ATPase or BF_1 from *M. lysodeikticus* (strain A PWYE, Carreira et al., 1976) was purified by preparative polyacrylamide gel electrophoresis as already reported (Andreu & Muñoz, 1975). Its two major subunits were isolated by preparative polyacrylamide gel electrophoresis in the presence of 8 M urea–1 mM dithiothreitol as described in detail elsewhere (Andreu et al., 1976). Time and temperature in 8 M urea were kept to a minimum and the purified subunits transferred to 6 M Gdn·HCl in 50 mM Tris-HCl, 1 mM dithiothreitol (pH 7.5) by extensive ultrafiltration. Subsequent storage was at −20 °C.

The homogeneity of the protein preparation was tested by analytical gel electrophoresis carried out at different pH values and acrylamide concentrations (see below). These experiments allowed us to conclude that BF_1 was more than 98% pure and the α and β subunits were more than 92% pure. As an example, Figure 1 illustrates the densitometric scans obtained in some of those experiments. The amino acid composition and sugar content of *M. lysodeikticus* BF_1 and its α and β subunits as well as the lability of the α subunit have already been described (Andreu et al., 1976).

Protein concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard, from the extinction coefficients of purified proteins (see Results) or by amino acid analysis (Andreu et al., 1973, 1976).

Analytical Polyacrylamide Gel Electrophoresis. The two discontinuous buffer systems made up of Hepes–imidazole/imidazole hydrochloride (pH 7.2) and Tris–glycine/Tris-HCl (pH 8.4) described previously (Andreu et al., 1976) were used for analytical electrophoresis. Both systems were applied under native conditions, in the presence of mild detergents (0.1% Triton X-100, 0.1–0.5% sodium deoxycholate) or supplemented with 0.1% $NaDodSO_4$ or 8 M urea–1 mM dithiothreitol. Different acrylamide concentrations were used, the acrylamide/bisacrylamide ratios being kept constant. Ferguson plots (relative mobility vs. acrylamide concentration) were constructed from the experimental data obtained, according to Rodbard & Chrambach (1971), to estimate the molecular weights of purified proteins by comparison with standard proteins, taking advantage of the relationship between molecular size and retardation coefficient.

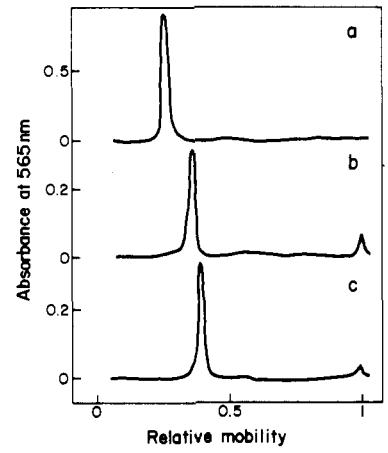

FIGURE 1: Purity of holo-ATPase of *Micrococcus lysodeikticus* and its isolated α and β subunits checked by gel electrophoresis in Hepes–imidazole/imidazole hydrochloride (pH 7.2). (a) Electrophoresis in 7% acrylamide and native conditions of holo-ATPase (15 μg of protein); (b and c) electrophoresis in 10% polyacrylamide–sodium dodecyl sulfate of α (8 μg) and β (8 μg), respectively.

Proteins were stained with Coomassie blue R250 (Andreu et al., 1973) or, occasionally, in native or urea gels, with Coomassie G250 following the procedure by Diezel et al. (1972).

Gel Chromatography in Guanidine Hydrochloride. Sepharose 6B was equilibrated and eluted at room temperature with 6 M Gdn·HCl in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol. A column of 0.9 × 55 cm was used and the void (V_0) and inclusion ($V_o + V_i$) volumes were estimated from the elution positions of blue dextran and tryptophan, respectively. These two markers were dissolved in elution buffer together with the sample and run into the column when an accurate determination of elution parameters was desired. The solvent volumes were estimated by weighing the fractions of approximately 1 g. The flow rate was kept constant at 1 mL/h with the help of a Sage 375 A peristaltic pump and the effluent was continuously monitored at 206 and 278 nm with a double-beam Uvicord III (LKB) equipped with cells of 3-mm light path and a multichannel register to determine the elution volume (V_e) of samples.

Under these conditions, the following relation holds (Fish et al., 1970; Fish, 1975): $MW^{0.555} \simeq A + B\ \mathrm{erf}^{-1}(1 - K_d)$, where MW is the molecular weight of the random coil polypeptides, K_d is the partition coefficient ($K_d = (V_e - V_o)/V_i$), A and B are constants of the system and erf^{-1} is the inverse of the error function

$$\mathrm{erf}\,(y) = 2/\sqrt{\pi} \int_{x=1}^{x=y} e^{-x^2}\,dx$$

Partial Specific Volume. The values of the effective partial specific volume of BF_1 in aqueous buffer and in 6 M Gdn·HCl were measured experimentally by densimetry according to Lee & Timasheff (1974a) with a mechanical oscillator-type density meter Paar DMA 02 (Kratky et al., 1973) at 20 ± 0.02 °C and low macromolecule concentrations (0–3 mg/mL) measured by ultraviolet absorption of the protein moiety. The values of partial specific volume were calculated from the chemical composition, i.e., amino acid and sugar content (Fish, 1975), of *M. lysodeikticus* BF_1 and its α and β subunits (Andreu et al., 1976). The values of the effective partial specific volume in 6 M Gdn·HCl were calculated from the amino acid composition of the proteins taking into account

Table I: Partial Specific Volumes of *M. lysodeikticus* ATPase and Its α and β Subunits

		partial sp vol[a] (mL·g^{-1})		
		calcd from AA composition	calcd from AA + sugar composition	measd by densimetry
ATPase (BF_1)	50 mM Tris-HCl (pH 7.5)	0.733	0.719	0.715 ± 0.010
α	50 mM Tris-HCl (pH 7.5)	0.731	0.717	
β	50 mM Tris-HCl (pH 7.5)	0.730	0.723	
ATPase (BF_1)	50 mM Tris-HCl, 6 M Gdn·HCl (pH 7.5)	0.730	0.716[b]	0.722 ± 0.005
α	50 mM Tris-HCl, 6 M Gdn·HCl (pH 7.5)	0.733	0.719[b]	
β	50 mM Tris-HCl, 6 M Gdn·HCl (pH 7.5)	0.729	0.722[b]	

[a] See Materials and Methods for calculation and measurements of density and concentration of protein solutions. AA, amino acid. [b] These three figures should be considered as rough estimates because the possible contribution of preferential interaction of the sugar residues with the solvent has not been taken into account.

the preferential interaction with the solvent (Lee & Timasheff, 1974b). Table I summarizes these results.

Owing to the low amounts of purified α and β subunits, their partial specific volumes could not be measured directly. On the basis of the determinations recorded in Table I and because α and β represent most of the mass of the BF_1 molecule (Andreu et al., 1973), we took a value of 0.72 mL/g as the effective partial specific volume of α and β (glyco) polypeptides in 6 M Gdn·HCl and assumed it to involve an error smaller than 0.005 mL/g.

Sedimentation Equilibrium. These experiments were performed according to the meniscus depletion method of Yphantis (1964), by using a Spinco Model E analytical ultracentrifuge equipped with the Rayleigh interference optical system. Samples were dissolved in 50 mM Tris-HCl, 6 M Gdn·HCl (pH 7.5) containing 1 mM dithiothreitol and reference solutions carefully prepared from ultrafiltrates of samples in order to match their refractive indexes. The height of the liquid column was 3 mm. Attainment of equilibrium was assessed 20 h after starting the runs by taking photographs at 1-h intervals. For samples showing a limited degree of heterogeneity, the results were analyzed in terms of a mixture of two molecular species by application of the two species plot (Roark & Yphantis, 1970); the methodology of Teller (1973) was followed. The average reduced molecular weights (weight average σ_w and number average σ_n) were determined at several distances, r, from the center of rotation. The relation $\sigma_w(r) = -\sigma_1\sigma_2|1/\sigma_n(r)| + \sigma_1 + \sigma_2$ holds for a mixture of two molecular species 1 and 2. By plotting $\sigma_w(r)$ against $1/\sigma_n(r)$, a straight line is obtained, intersecting at points $(\sigma_1, 1/\sigma_1)$ and $(\sigma_2, 1/\sigma_2)$, the hyperbola obtained by representing σ vs. $1/\sigma$ values. A concave plot may result from the presence of more than two species while a convex plot would suggest thermodynamic nonideality (Teller, 1973).

Charge Shift Electrophoresis. The procedure was that described by Helenius & Simmons (1977). Electrophoreses were performed in 1% agarose on glass plates (8 × 9 cm) using a water-cooled chamber (Multiphor 2117 LKB) and 50 mM glycine/NaOH–100 mM NaCl (pH 9.0) or 100 mM imidazole hydrochloride (pH 7.2) as buffer systems. The following detergents were used: 0.5% Triton X-100, 0.25% sodium deoxycholate, and 0.05% cetyltrimethylammonium bromide. Runs were performed at 4 V cm^{-1} for 45 or 60 min.

Light Absorption. Routine measurements of absorption were carried out on a Gilford 2400 spectrophotometer. Ultraviolet spectra were recorded with a double beam Beckman Acta C III spectrophotometer at 20 °C using bandwidths smaller than 1 nm. The spectrophotometer was checked with absorption and wavelength standards.

The protein extinction coefficients in denaturing solvents (50 mM Tris-HCl, 6 M Gdn·HCl, pH 7.5, 1 mM dithiothreitol) were calculated from the absorbance values and the protein concentration estimated by amino acid analysis (Andreu et al., 1973, 1976). The protein spectra in neutral aqueous buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol) were corrected for light scattering by extrapolation of the absorbances within the 320–360-nm range (Winder & Gent, 1971). Proteins were transferred from denaturing conditions to aqueous buffer by 100–500-fold dilution and back again to 6 M Gdn·HCl by adding the required amount of solid guanidine hydrochloride. The dilution factors were taken into account in each case and Gdn·HCl concentration was measured by refractometry (Nozaki, 1972). The dilute protein solutions (about 10–100 μg/mL aqueous buffer) underwent an apparent loss (never exceeding 30% of the total) of light absorbing material which might be due to precipitation or adsorption to the glass walls. It was assumed that this did not occur in 6 M Gdn·HCl and this assumption was used to correct the values of protein concentration. As a matter of fact, the extinction coefficients in aqueous buffer reported here were derived from the extinction coefficients obtained in 6 M Gdn·HCl and the absorption ratios of the proteins transferred from aqueous buffer to 6 M Gdn·HCl. The values for these ratios (buffer/6 M Gdn·HCl) were, at 276 nm respectively, 1.22 for α, 1.02 for β, and 1.25 for whole BF_1.

All the optical properties were referred to the protein moieties. In order to calculate the values corresponding to the whole glycoprotein molecules, the following carbohydrate content has to be considered: 0.06 g/g of peptide for α, 0.12 g/g of peptide for β, and 0.12 g/g of peptide for holo-BF_1 (Andreu et al., 1976, 1978).

Fluorescence. Fluorescence measurements were performed with a Fica 55 MKII difference spectrofluorimeter which recorded the excitation and emission spectra corrected for lamp quantum output and detector response. The apparatus was calibrated with quinine sulfate, tyrosine, and tryptophan. The excitation and emission bandwidths were set at 2.5 and 7.5 nm, respectively. Cuvettes (0.5 × 0.5 cm) were thermostated at 20 ± 0.5 °C. The exciting light absorption of the samples was kept below 0.01 to reduce inner filter effects. The exposure of the proteins to the excitation beam was minimized to avoid photolysis. The intensities of emission (relative quanta output) were standardized to 1 g/L of protein concentration by means of arbitrary units (signal output in volts using a reference photomultiplier voltage of 600 V and a measuring gain factor of 50).

Circular Dichroism. Circular dichroism spectra were obtained at a sample temperature of 20 ± 0.5 °C with a Rousell-Jouan 18 J dichrograph II calibrated with epiandrosterone. The bandwidths were smaller than 1 mm and cuvettes of 1, 0.5, and 0.2 cm were used. The concentration of the samples was such as to obtain the lowest amount of scattering possible. The values of differential dichroic ab-

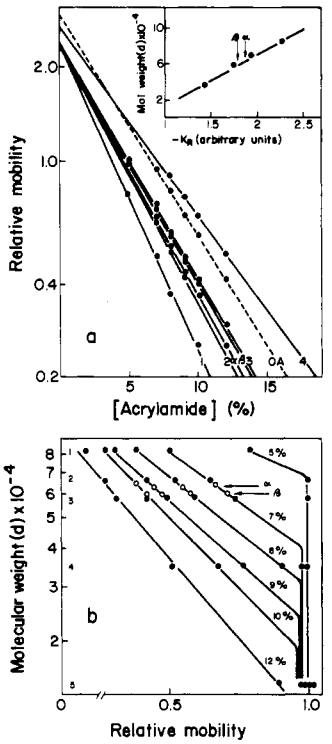

FIGURE 2: (a) Ferguson plots of isolated α and β subunits of *Micrococcus lysodeikticus* ATPase as compared with those of standard proteins and a standard glycoprotein (egg albumin, OA). The inset shows the relationship between the plot slope and molecular weight. Electrophoresis was carried out in $NaDodSO_4$–polyacrylamide gels (pH 7.2) (see Materials and Methods). (b) Calibration of the $NaDodSO_4$–polyacrylamide system at pH 7.2 for gels of 5–12% acrylamide concentration. (○) α and β subunits; (●) standard proteins. (1) Urease; (2) bovine serum albumin; (3) catalase; (4) lactic dehydrogenase; and (5) lysozyme.

sorption ($A_L - A_R$) were converted into mean residue molar ellipticity [θ] by means of the relation: $[\theta] = 3300(A_L - A_R)/cd$, deg·cm²·dmol⁻¹ (Adler et al., 1973), where c was the mean residue concentration in mol/L and d the light path in cm. The value of c was calculated from the protein concentration and the mean residue weight deduced from the amino acid composition (Andreu et al., 1976).

Results

Molecular Weight of Monomeric, Denatured α and β Subunits. These experiments were designed to obtain the most reliable molecular weights of the two subunits. This is a prerequisite for a full molecular characterization of *M. lysodeikticus* ATPase and for a correct estimation of its subunit stoichiometry.

Molecular weights were estimated in the presence of three denaturants: 8 M urea, 0.1% $NaDodSO_4$, and 6 M guanidine hydrochloride using different techniques such as polyacrylamide gel electrophoresis, gel chromatography, and sedimentation equilibrium. Isolated α and β subunits were fully unfolded in 6 M Gdn·HCl and 8 M urea as deduced from their optical properties (see below).

Figure 2a illustrates the Ferguson plot analysis of sodium dodecyl sulfate gel electrophoresis of isolated α and β subunits as compared with standard proteins. The lines of the two subunits and standard proteins intersect close to the ordinate axis indicating normal behavior for α and β as found in nonmodified proteins. This behavior contrasted with that of egg albumin, a standard glycoprotein, and was striking in view of the glycoprotein nature of α and β subunits (Andreu et al., 1976). A possible explanation for this discrepancy is given under the Discussion section. From the empirical relationship between the retardation coefficient and molecular weight (see inset of Figure 2a), the following molecular weights were calculated: α, 63 000 ± 3000, and β, 58 000 ± 3000. By plotting the relative mobility of the proteins at different acrylamide concentrations against molecular weight (Figure 2b), values of 61 500 ± 2000 and 58 000 ± 1500 were calculated for α and β subunits, respectively.

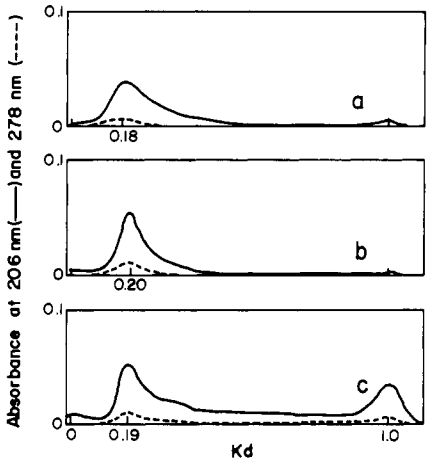

FIGURE 3: Sepharose 6B gel chromatography of α (a), β (b), and ATPase (c) in 6 M Gdn·HCl. The experimental conditions are described under Materials and Methods. The amount of protein charged on the columns was respectively 300 μg of α and β and 600 μg of holo-ATPase. As indicated in the ordinate axis, dashed lines represent the absorbance at 278 nm and dark lines represent the increased absorbance at 206 nm. The columns were calibrated in 6 M Gdn·HCl with the following standard proteins: phosphorylase *a*, urease, bovine serum albumin, catalase, lactic dehydrogenase, and trypsin.

The retardation coefficients of α and β subunits in 8 M urea, pH 7.2, were very close (not shown here; Andreu & Muñoz, 1975). Roughly estimated molecular weights under these experimental conditions were 54 000 ± 10 000 (α) and 53 000 ± 10 000 (β).

Figure 3 illustrates the elution profiles of ATPase, α and β in Sepharose 6B, 6 M Gdn·HCl. In this system, isolated α and β subunits appear to be more than 95% homogeneous, although a certain asymmetry of the subunit peaks is also observed. This fact and the small amount of low molecular weight material at $K_d = 1$ may result from intrinsic autodegradation of *M. lysodeikticus* BF_1 polypeptides as already reported (Nieto et al., 1975; Carreira et al., 1976; Andreu et al., 1976). From the K_d values of α (0.18) and β (0.20) and the calibration of the system, the following molecular weights were deduced: α, 57 500 ± 2200, and β, 53 200 ± 2200.

In the high-speed sedimentation–equilibrium experiments, very high rotor speeds, about 50 000 rev/min, were required to achieve good meniscus depletion of α and β solutions in 6 M Gdn·HCl, a requisite for the correct application of the Yphantis method (1964). The plot of ln c vs. r^2 did not markedly deviate from linearity. Nevertheless, the apparent molecular weights were strikingly low (<25 000) and changed as a function of the rotor speed. The average molecular weights calculated at each point varied as a function of the radius value. This was an indication of heterogeneity. Assuming that this was due to the presence of a limited amount of low molecular weight material in the subunit preparations as shown by gel chromatography experiments (see above), the two-species plot was applied; the results corroborated the assumption. Figure 4 illustrates the treatment which allowed

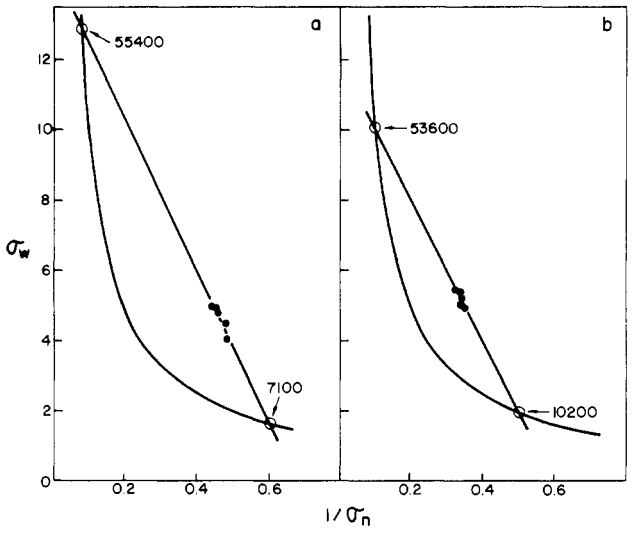

FIGURE 4: Two-species plot of the data of high-speed equilibrium–sedimentation experiments in 6 M Gdn·HCl of α and β subunits. Initial protein concentration was about 0.3 mg/mL in both cases. Rotor speeds and run temperatures were 52 419 rpm and 1 °C for α (a) and 48 327 rpm and 3 °C for β (b).

Table II: Summary of the Molecular Weight Values of the Two Major Subunits of *M. lysodeikticus* ATPase as Estimated by Different Methods

method of determination[a]	mol wt[b] α	mol wt[b] β
$NaDodSO_4$-polyacrylamide gel electrophoresis (pH 7.2)	62 200 ± 2500	58 000 ± 2400
Sepharose 6B gel chromatography in 6 M Gdn·HCl	57 500 ± 2200	53 200 ± 2200
sedimentation equilibrium in 6 M Gdn·HCl	55 400 ± 8600	53 600 ± 7700

[a] A detailed analysis of the treatment of the experimental data is given under the Materials and Methods and Results sections. [b] Values were obtained in each case by averaging or linear least-squares analysis. Standard deviations are shown, except for the sedimentation equilibrium experiments, where confidence limits for 75% probability were calculated by means of student's t distribution owing to the large extrapolation involved (see Figure 4).

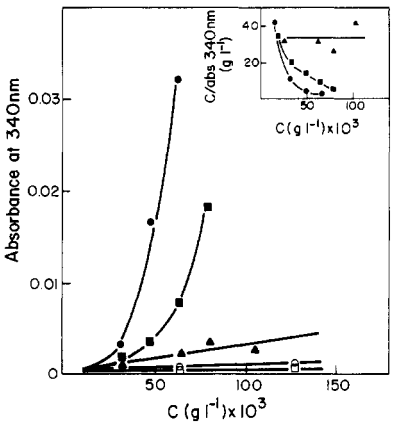

FIGURE 5: Absorbance at 340 nm (turbidity) vs. protein concentration: α (○) and β (■) subunits in 50 mM Tris-HCl (pH 8.0); α (□) and β (●) in 50 mM Tris-HCl supplemented with 0.5 M NaCl; β (▲) in 50 mM Tris-HCl plus 0.1% sodium deoxycholate. The inset shows the concentration dependence of light scattering of β in the different solvents to illustrate the type of intermolecular interactions according to Timasheff & Townnend (1970).

us to calculate a 55 400 ± 8600 molecular weight for α and 53 600 ± 7700 for β. The minor components had similar molecular weights in both cases (7000 to 10 000).

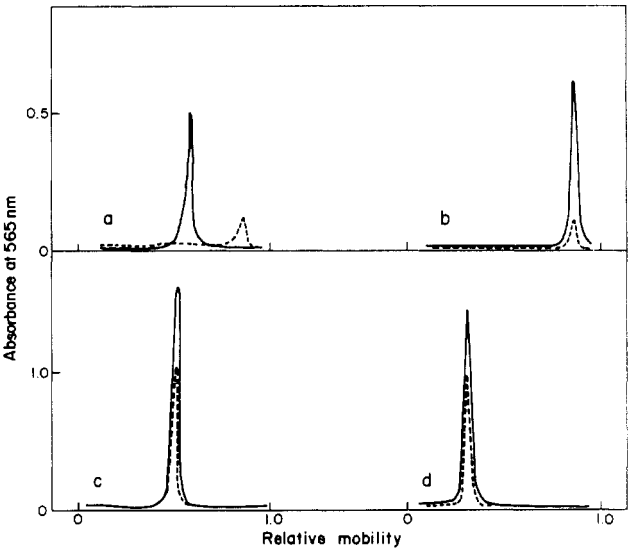

FIGURE 6: Densitometric scans of the electrophoretic analysis of refolded β subunits (a and b) as compared with that of whole ATPase (c and d). (a and c) Electrophoresis in 6% acrylamide gels, 0.2 M Tris, pH 8.4, with 0.1% Triton X-100 (—) or without detergent (– – –); (b and d) electrophoresis in 7% acrylamide gels, 0.4 M Tris, pH 8.4, with 0.1% sodium deoxycholate (—) or without sodium deoxycholate (– – –). Samples contained 10 μg of protein in all cases.

Table II compares the molecular weights of α and β subunits obtained with the $NaDodSO_4$ and Gdn·HCl solvents.

Aggregation of α and β Subunits in Aqueous Buffers. Effect of Ionic Strength and Interaction with Detergents. In order to refold α and β subunits into native-like structures, the preparations were diluted in aqueous buffers to decrease denaturant concentration from 6 M to 10–50 mM. Both α and β in neutral aqueous buffer showed evident scattering in their ultraviolet spectra. Taking the absorption at 340 nm as an arbitrary parameter to measure light dispersion, qualitative differences in the state of molecular aggregation were observed. As shown in Figure 5, small scattering which is roughly proportional to protein concentration was observed for α solutions at medium ionic strength. Interestingly, the scattering increased after addition of sodium deoxycholate but decreased when the ionic strength of the medium increased. On the other hand, the β subunit in neutral aqueous buffer produced strong light scattering, both at low and medium ionic strength, which was not linearly dependent on protein concentration and increased at high ionic strength. The addition of Triton X-100 or sodium deoxycholate reduced the scattering contribution and made it linearly dependent on protein concentration. A treatment of these data according to a plot usually applied in light scattering studies (see inset of Figure 5) allowed us to conclude that β molecules interacted strongly and then aggregated. The aggregation was facilitated by the ionic strength of the medium but was reduced by mild detergents. It is worth noting that the aggregation of β did not interfere with the study of its optical properties (see below), even in the absence of detergent, provided that this study was carried out at a relatively low protein concentration. The deviation from the Beer–Lambert law at 275 nm of β solutions was less than 2% below 40 μg of protein/mL, although it increased rapidly beyond 50 μg of protein/mL.

The molecular state of the subunits was also examined by polyacrylamide gel electrophoresis in the absence of denaturants. By conventional staining procedures, only a relatively small proportion of the β subunit applied to the gels was seen in these experiments, whereas the α subunit could not be seen at all. If the electrophoreses were carried out in the presence

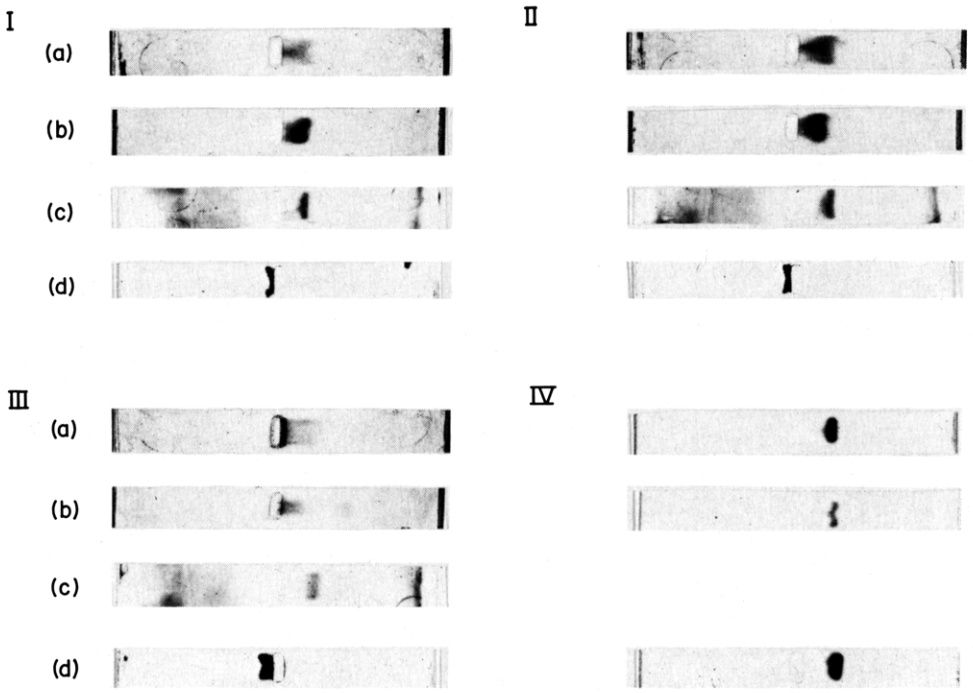

FIGURE 7: Charge shift electrophoresis of α (I), β (II), intrinsic membrane proteins of *M. lysodeikticus* (III), and egg albumin (IV) showing typical experiments at pH 9.0 of samples containing 5–10 μg of protein. (a) No detergent; (b) with Triton X-100; (c) Triton X-100 plus sodium deoxycholate; (d) Triton X-100 plus cetyltrimethylammonium bromide. Runs were for 60 min except in IV (45 min). Anode (+) was on the right side. The black lines in both sides are the edges of the plates. For other details, see Materials and Methods.

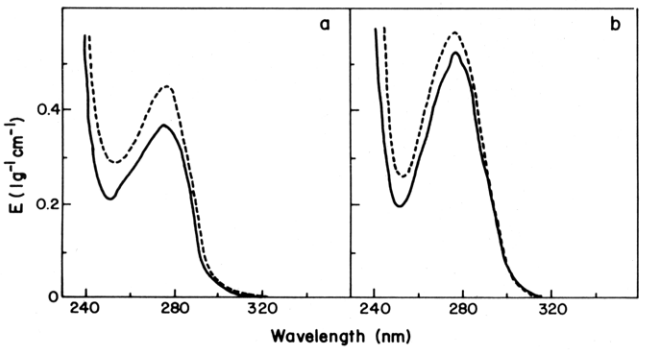

FIGURE 8: Absorption spectra of α (a) and β (b) subunits in 6 M Gdn·HCl (continuous line) and in aqueous buffer (broken line). The spectra were obtained from α solutions of protein concentrations ranging from 10 to 100 μg/mL and β solutions of concentrations from 10 to 50 μg of protein/mL.

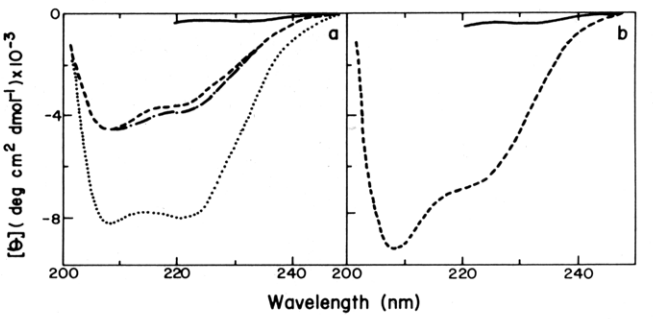

FIGURE 9: Circular dichroism spectra of α (a) and β (b) in 6 M Gdn·HCl (—), in 10–50 mM Tris-HCl, pH 8.0 (– – –), and in Tris buffer plus 0.5 M NaCl (—·—), as compared with the spectrum of holo-ATPase (···, a) in 50 mM Tris-HCl, pH 8.0. Sodium deoxycholate (0.1%) caused no significant changes in the β spectrum. Protein concentrations were as in Figure 8.

of 0.1% Triton X-100 or sodium deoxycholate, the amount of material showing positive staining increased for β in the form of a single band (see Figure 6), whereas the α subunit lacked stain or did not enter the gel.

Charge Shift Electrophoresis. In view of the above results, we applied this technique for a rapid diagnosis of hydrophobic domains in the folded α and β subunits. Figure 7 illustrates the behavior of α and β subunits suggesting their ability to bind mild detergents, as the intrinsic membrane proteins from *M. lysodeikticus* did, but unlike egg albumin, a soluble glycoprotein. Both α and β underwent an anodic shift with regard to their mobility in neutral aqueous detergent when run in neutral plus anionic detergents, but a cathodic shift when run in neutral plus cationic detergent. These results prompted us to investigate the behavior of holo-BF_1 in charge shift electrophoresis. The preliminary results suggest that whole BF_1–ATPase behaves like isolated α and β subunits. A detailed description of the interaction of BF_1 with detergents will be published elsewhere.

Optical Properties of α and β in 6 M Gdn·HCl and Aqueous Buffer. In a further attempt at a molecular characterization of the unfolded and (pseudo) renatured states of α and β subunits, we examined their optical properties and compared them with those of whole BF_1–ATPase. The spectra in denaturing conditions were obtained from protein solutions in 6 M Gdn·HCl, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol and those in native conditions from protein solutions in 50 mM Tris-HCl (pH 7.5 or 8.0), 1 mM dithiothreitol with a residual concentration of 10–50 mM denaturant. The omission of dithiothreitol did not influence the optical properties.

Figure 8 shows the ultraviolet absorption spectra of α and β subunits. In the presence of denaturant, both polypeptides showed maxima at 275.5 ± 0.5 nm, minima at 250 ± 1 nm, and shoulders at 258 ± 1 nm, 264 ● 1 nm, 269 ± 1 nm, and 290 ± 1 nm. In aqueous buffers, the maxima were at 276 ± 0.5 nm, minima at 253 ± 1 nm, and shoulders were detected at 258 ± 1 nm, 268 ± 1 nm, and 290 ± 1 nm. These properties in the two states are similar to those observed in the denatured and native holo-ATPase, respectively (Nieto et al., 1975). However, the absorption coefficients of α and β do not account for the quantitative absorption data of holo-ATPase. In 6 M

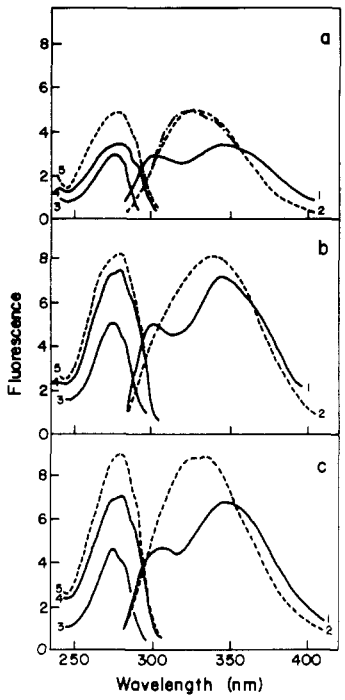

FIGURE 10: Fluorescence of α (a), β (b), and ATPase (c) in 6 M Gdn·HCl (continuous line) and in aqueous buffer (broken line); (—·—) illustrates the fluorescence of α subunit in aqueous buffer plus 0.5 M NaCl. Sodium deoxycholate (0.1%) caused no significant changes in the fluorescence of β. Curves 1 and 2 represent the emission spectra (excitation was at 275 nm); curves 3, 4, and 5 correspond to the excitation spectra analyzed at each maximum for emission. Fluorescence was standardized to arbitrary units (see Materials and Methods). Protein concentration was below 100 μg/mL.

Table III: Fluorescence of ATPase of *M. lysodeikticus* and Its α and β Subunits. Position and Intensity of the Emission Maxima by Excitation at 275 nm

solvent	wavelength of maxima (nm)	emission intensity (arbitrary units)		
		ATPase	α	β
50 mM Tris-HCl, 6 M Gdn·HCl (pH 8.0)	301		2.8	4.8
	304	4.4		
	348	6.7	3.4	6.9
50 mM Tris-HCl (pH 8.0)	325	8.6		
	328		4.9	
	336	8.7		
	340			8.0

Gdn·HCl, the E_{276} values were 0.68 (whole ATPase), 0.37 (α), and 0.52 (β) L·g^{-1}·cm^{-1}, while in aqueous buffer they were 0.85 (ATPase), 0.45 (α), and 0.53 (β) L·g^{-1}·cm^{-1}.

The circular dichroic spectra of α and β in 6 M Gdn·HCl (see Figure 9) were featureless between 220 and 250 nm like that of ATPase under the same conditions. This is a characteristic of random coil polypeptides (Tanford, 1968). Urea (8 M) produced the same results. In aqueous buffer, α and β folded into structures with a higher order showing negative extrema at 208 ± 2 and 222 ± 2 nm similarly to native ATPase (see Figure 10a). The ellipticity value $[\theta]_{222}$ of α in Tris-HCl was −3500, as compared with a value of −6350 for β and −8200 deg·cm^2·dmol^{-1} for holo-ATPase. The $[\theta]_{208}$ values were −4350 (α), −8900 (β), and −8300 (ATPase) deg·cm^2·dmol^{-1}.

Figure 10 illustrates the fluorescence spectra of isolated α, β, and whole ATPase in the presence of denaturants and in aqueous buffer. In the presence of denaturant, α and β showed two well-separated emission maxima at 301 ± 1 nm and 348 ± 1 nm corresponding, respectively, to tyrosine and tryptophan exposed to the solvent (Chen et al., 1969). A similar spectrum was obtained for the ATPase in Gdn·HCl. The excitation spectra of the two maxima mimicked the absorption spectra of free tyrosine and tryptophan. For instance, the emission spectra of β solutions could be simulated by a linear combination of the emissions of its tyrosine and tryptophan content as free amino acids with a quenching factor of 2.8-fold and 1.3-fold, respectively.

The emission spectra of both α and β in aqueous neutral buffers underwent a marked change showing the disappearance of the maxima corresponding to tyrosine and increase and shift of tryptophan emission to 328 ± 1 (α) and 340 ± 1 nm (β). This observation strongly suggests a location of the tryptophan residues in a more hydrophobic domain than the solvent (Chen et al., 1969). A similar result, consisting in an emission spectrum with two maxima at 325 ± 1 and 336 ± 1 nm, was observed with native holo-ATPase. Nevertheless, the fluorescence of α and β together did not account for the fluorescence of the whole protein. Table III summarizes the quantitative results.

Discussion

This report represents the first study of its type on the major polypeptides of an energy-transducing ATPase, one of the most complex enzyme systems found in nature. The reconstitution of ATPase activity from *E. coli* subunit complexes (Vogel & Steinhart, 1976), and isolated subunits from the BF_1 of PS_3 thermophile bacterium (Yoshida et al., 1977a,b), has been reported. Furthermore, the reconstitution of ATPase activity from isolated subunits of *E. coli* BF_1 (Futai, 1977) has also been described. However, these studies did not attempt to describe the molecular properties of the isolated subunits or the reconstituted complexes. ATPase activity of *E. coli* BF_1 was reconstituted from seemingly native-like structures, while reconstitution of BF_1 from the thermophile PS_3 could involve the refolding and reassociation of subunits since these polypeptides were exposed to 8 M urea. However, this BF_1 showed an unusual resistance to denaturing conditions (i.e., 50% of its ATPase activity was maintained in 5.5 M urea; Yoshida et al., 1975) and it, therefore, seemed likely that subunits from BF_1 of PS_3 were not fully unfolded in 8 M urea. This may imply the presence of a residual native structure that facilitates partial refolding and reassociation upon removal of denaturant. This point remains unanswered because data on the secondary structure of isolated subunits from BF_1 of PS_3 were not reported by Yoshida et al. (1977a,b). The present study was aimed at characterizing isolated α and β subunits of *M. lysodeikticus* BF_1. We carefully determined the molecular weights of their random coil forms in 6 M Gdn·HCl and examined their refolding and aggregation when the denaturant was removed.

It has been possible to estimate reliable molecular weights of the major subunits. The differences found, depending on the method used, deserve comment. The $NaDodSO_4$–polyacrylamide gel system rendered the highest molecular weight values. This may be consistent with the glycoprotein nature of the two subunits, though their behavior in this analytical system corresponded to that of nonmodified proteins (see Results). A possible explanation for this discrepancy is given below. On the other hand, the molecular weights obtained using 6 M Gdn·HCl should not suffer the possible drawbacks of the molecular weights obtained from the $NaDodSO_4$–polyacrylamide gel system, because it has been shown that glycoproteins with less than 20% carbohydrate content behave

like nonmodified proteins in 6 M Gdn·HCl (Fish, 1975). The sedimentation equilibrium method presented the advantage of being independent of the presence of standards as external references. Nevertheless, the treatment of the data according to ln concentration against the squares of radial distance was apparently incorrect, as inconsistent values were obtained. The interpretation in terms of the presence of two species, the intact subunit and a small percentage of low molecular components, seems satisfactory and furthermore agrees with the observation by Jeffrey & Pont (1969) who showed that artificial mixtures of serum albumin (mol wt 67 000) or egg albumin (44 500) with 5% (w/v) ribonuclease (13 400) gave molecular weight estimations of about 20 000–35 000 by the Yphantis method. Therefore, the determinations of the molecular weight of α and β subunits by a straightforward application of this technique may be incorrect owing to the presence of components resulting from subunit autodegradative processes related to their glycoprotein nature (Andreu et al., 1976). The same might be true for the molecular weights of the whole protein estimated by this technique. This interpretation may explain the results obtained by Schnebli et al. (1970), who found subunit molecular weights of 30 000 for the urea-denatured ATPase of *Streptococcus faecalis*, using appropriate speeds as well as the apparent correct values obtained for mitochondrial F_1 subunits (Knowles & Penefsky, 1972). If mitochondrial F_1 polypeptides undergo a similar degradative process, the last observation may result from the fortuitous combination of two facts, sample heterogeneity and overestimation of the molecular weight by the use of excessively low speeds.

Keeping in mind the limitations of the method that are evident from these same studies, the stoichiometry of *M. lysodeikticus* ATPase can be examined under a better defined experimental perspective in the light of the present data on the molecular weight of the two major subunits. Furthermore, a molecular weight of 34 000 ± 4500 for the γ subunit has also been determined (Andreu, J. M., & Muñoz, E., unpublished results). The molecular weight 345 000 determined previously for the whole protein (Andreu et al., 1973) can be taken as a minimum value because it may be subjected to the experimental drawbacks induced by the presence of some low molecular weight components (see above). Assuming average molecular weights of 58 400 ± 5300 (α) and 54 900 ± 4800 (β), molecular formulas like $\alpha_2\beta_2\gamma$ (260 600 ± 11 100) and $\alpha_2\beta_2\gamma_2$ (294 600 ± 11 900) are highly improbable by defect, whereas $\alpha_4\beta_4\gamma$ (487 200 ± 15 300) and $\alpha_4\beta_4\gamma_2$ (521 200 ± 15 700) are highly improbable by excess. Subunit stoichiometries $\alpha_3\beta_3\gamma$ (373 900 ± 13 200) and $\alpha_3\beta_3\gamma_2$ (407 900 ± 13 900) seem more likely. A careful determination of the molecular weight of ATPase is critical to settle the question. Preliminary sedimentation equilibrium experiments at low speed carried out in this laboratory indicate a molecular weight close to 430 000. Moreover, the consideration of hydrodynamic parameters of BF_1, like the sedimentation coefficient of 13.6 S (Andreu et al., 1973) and the radius of the equivalent sphere (75 Å) determined by gel chromatography (Andreu, J. M., unpublished results), also supports molecular weights of over 400 000. These data favor stoichiometry of the $\alpha_3\beta_3\gamma_{1-2}$ type, but it may be taken with caution until results with other experimental approaches, in particular on the crystallography of the protein, are available. In this context, it is worth noting that recent work on the crystallography of the ATPases from mitochondria (Amzel & Pedersen, 1978) and the termophile bacterium PS_3 (Wakabayashi et al., 1977) showed an axis of symmetry that seems difficult to reconcile with the stoichiometry suggested by us. However, the conclusions from that crystallographic work were not so obvious. In one case, Wakabayashi et al. (1977) proposed a stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ for the bacterial ATPase and Amzel & Pedersen (1978) considered it premature to speculate about possible subunit stoichiometries of mitochondrial F_1.

As shown by light scattering and polyacrylamide gel electrophoresis, α and β subunits aggregated in neutral aqueous buffer, though differences between the aggregation processes of each subunit were evident. The moderate aggregation of α may involve ionic interactions because it was decreased by salt. The massive aggregation of β was increased by high ionic strengths and prevented by the use of mild detergents. These observations suggest that the aggregation of β subunits may occur through hydrophobic interactions. This apparently stronger hydrophobic character of the β subunit than that of the α subunit of *M. lysodeikticus* BF_1 contrasts with the observations by Futai (1977) on *E. coli* ATPase. However, subunit nomenclature is based on the mobility on $NaDodSO_4$ polyacrylamide gel electrophoresis and a possible functional crossing of α and β subunits of *E. coli* ATPase has been suggested (Verheijen et al., 1978). Another important novel structural feature of soluble *M. lysodeikticus* BF_1, α and β, is their apparently amphiphilic character as revealed by the charge shift electrophoresis experiments. To our knowledge, this is the first report on this property, its interest being due to the glycoprotein nature of the BF_1–ATPase and its generally accepted classification as an extrinsic membrane protein. This property may by highly relevant to the assembly of the protein and its interaction with the membrane. On the other hand, the amphiphilic character of the α and β subunits may be accompanied by an increase in $NaDodSO_4$ binding, unfavored by their carbohydrate content, and thus facilitate standard binding of the detergent. This would explain their apparently normal behavior in $NaDodSO_4$–polyacrylamide gel electrophoresis (see above).

Random coil α and β subunits are able to refold in forms showing secondary structures with a significant degree of order (β more than α), though they do not coincide with that of the native ATPase. It must be noted that we have not taken into account the contribution of the sugar residues to circular dichroism spectra. This contribution must be low since it is less than 10% of the amplitude within the measured wavelength range in glycoproteins containing up to 40% by weight of carbohydrates like the α_1 acid glycoprotein (Aubert & Loucheux-Lefebre, 1976). In 6 M Gdn·HCl, both α and β show fluorescence spectra with well-separated emissions of tryptophan and tyrosine, suggesting a decrease of tyrosine quenching and/or decrease of energy transfer from tyrosine to tryptophan (Chen et al., 1969). Tryptophan and tyrosine of the same subunit show separate fluorescence in 6 M Gdn·HCl. Thus, it is not necessary to postulate the presence of subunits lacking tryptophan (Nieto & Ayala, 1977) to account for the two emission maxima observed in whole protein denatured with Gdn·HCl. In aqueous buffer, α and β seem to place their fluorophores in more hydrophobic domains (Chen et al., 1969) similar to those of native ATPase as judged by the disappearance of the maximum of the emission corresponding to tyrosine and the blue shift of tryptophan to wavelengths near the two emission maxima of native ATPase.

In summary, the optical properties of refolded α and β were qualitatively similar to the properties of native ATPase but the average values of α plus β were smaller than the corresponding average properties of native BF_1, although α plus β represent most of the mass of the BF_1 molecule (Andreu et

al., 1973). The same discrepancy was even observed between the absorption and fluorescence of denatured ATPase and its random coil α and β subunits. These observations can be explained by the following causes: (i) intersubunit interactions existing in native ATPase, thus not present in isolated subunits; (ii) the presence in BF_1 of minor components enriched in optically active residues (i.e., components of relative mobility 1 in $NaDodSO_4$ gel electrophoresis at pH 8.4 and 7% acrylamide; Andreu et al., 1973). In this respect, it is worth noting that the tyrosine, tryptophan, and sugar contents of α plus β do not account for those of holo-BF_1 (Andreu et al., 1976).

The presence of sugars covalently bound to *M. lysodeikticus* BF_1 polypeptides (Andreu et al., 1978) might also restrict the ability to refold properly from the random coil state. It has been stated that glycosylation seems to start on nascent polypeptide chains (Rothman & Lodish, 1977) and to continue later on (Waechter & Lennarz, 1976). Since the primary folding processes are relatively rapid (Anfinsen & Scheraga, 1975), it follows that conformational constraints by added oligosaccharide may be imposed on partially or completely folded polypeptide chains. Thus after unfolding, the glycopeptides might be unable to find the exact folding pathway again. A similar suggestion has been made recently (Ayala & Nieto, 1978).

Refolded α, β subunits did not show ATPase activity. Work is in progress to define the ability to bind ligands (e.g., ADP) of isolated α, β, or different complexes of these subunits or with other components of BF_1. The obtention of active oligomers and the extension of this type of work and its application to other F_1 ATPases would be, in our opinion, the most conclusive approach for the understanding of the structure–function relationships of energy-transducing ATPases.

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References

Abrams, A., & Smith, J. B. (1974) *Enzymes, 3rd Ed. 10*, 395–429.
Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675–735.
Amzel, L. M., & Pedersen, P. L. (1978) *J. Biol. Chem. 253*, 2067–2069.
Andreu, J. M., & Muñoz, E. (1975) *Biochim. Biophys. Acta 387*, 228–233.
Andreu, J. M., Albendea, J. A., & Muñoz, E. (1973) *Eur. J. Biochem. 37*, 505–515.
Andreu, J. M., Carreira, J., & Muñoz, E. (1976) *FEBS Lett. 65*, 198–203.
Andreu, J. M., Warth, R., & Muñoz, E. (1978) *FEBS Lett. 86*, 1–5.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205–300.
Aubert, J. P., & Loucheux-Lefebre, M. H. (1976) *Arch. Biochem. Biophys. 175*, 400–409.
Ayala, J. A., & Nieto, M. (1978) *Biochem. J. 169*, 371–380.
Baird, B. A., & Hammes, G. G. (1977) *J. Biol. Chem. 252*, 4743–4748.
Carreira, J., Andreu, J. M., Nieto, M., & Muñoz, E. (1976) *Mol. Cell. Biochem. 10*, 67–76.
Chen, R. F., Edelhoch, H., & Steiner, R. F. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S., Ed.) Part A, pp 171–244, Academic Press, New York.
Diezel, W., Kopperschlager, G., & Hoffman, E. (1972) *Anal. Biochem. 48*, 617–620.
Estrugo, S. F., Larraga, V., Corrales, M. A., Duch, C., & Muñoz, E. (1972) *Biochim. Biophys. Acta 255*, 960–973.
Fish, W. W. (1975) *Methods Membr. Biol. 4*, 189–269.
Fish, W. W., Reynolds, J. A., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5166–5168.
Futai, M. (1977) *Biochem. Biophys. Res. Commun. 79*, 1231–1237.
Helenius, A., & Simmons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 529–532.
Jeffrey, P. D., & Pont, M. J. (1969) *Biochemistry 8*, 4597–4605.
Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6624–6630.
Kratky, O., Leopold, H., & Stabinger, H. (1973) *Methods Enzymol. 27*, 98–110.
Lee, J. C., & Timasheff, S. N. (1974a) *Biochemistry 13*, 257–265.
Lee, J. C., & Timasheff, S. N. (1974b) *Arch. Biochem. Biophys. 165*, 268–273.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mirsky, R., & Barlow, V. (1972) *Biochim. Biophys. Acta 291*, 480–488.
Muñoz, E., Salton, M. R. J., & Ellar, D. J. (1970) *FEBS Symp. 20*, 51–58.
Nelson, N. (1976) *Biochim. Biophys. Acta 456*, 314–338.
Nelson, N., Deters, D. W., Nelson, H., & Racker, E. (1973) *J. Biol. Chem. 248*, 2049–2055.
Nieto, M., & Ayala, J. (1977) *Biochem. J. 161*, 321–331.
Nieto, M., Muñoz, E., Carreira, J., & Andreu, J. M. (1975) *Biochim. Biophys. Acta 413*, 394–414.
Nozaki, Y. (1972) *Methods Enzymol. 26*, 43–50.
Pedersen, P. L. (1975) *J. Bioenerg. 6*, 243–275.
Penefsky, H. S. (1974) *Enzymes, 3rd Ed. 10*, 375–394.
Roark, D. E., & Yphantis, D. A. (1970) *Ann. N.Y. Acad. Sci. 164*, 245–278.
Rodbard, D., & Chrambach, A. (1971) *Anal. Biochem. 40*, 95–134.
Rothman, J. E., & Lodish, H. F. (1977) *Nature (London) 269*, 775–780.
Schnebli, H. P., Vatter, A. E., & Abrams, A. (1970) *J. Biol. Chem. 245*, 1122–1127.
Senior, A. E. (1973) *Biochim. Biophys. Acta 301*, 249–277.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Teller, D. C. (1973) *Methods Enzymol. 27*, 346–444.
Timasheff, S. N., & Townnend, R. (1970) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part B, pp 147–212, Academic Press, New York.
Verheijen, J. H., Postma, P. W., & Van Dam, K. (1978) *Biochim. Biophys. Acta 502*, 345–353.
Vogel, G., & Steinhart, R. (1976) *Biochemistry 15*, 208–216.
Waechter, C. J., & Lennarz, W. J. (1976) *Annu. Rev. Biochem. 45*, 95–112.
Wakabayashi, T., Kubota, M., Yoshida, M., & Kagawa, Y. (1977) *J. Mol. Biol. 117*, 515–519.
Winder, A. F., & Gent, W. L. G. (1971) *Biopolymers 10*, 1243–1251.
Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1975) *J. Biol. Chem. 250*, 7910–7916.
Yoshida, M., Okamoto, H., Sone, N., Hirata, H., & Kagawa, Y. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 936–940.
Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977b) *J. Biol. Chem. 252*, 3480–3485.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.