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Effect of Hydrostatic Pressure on the Mitochondrial ATP Synthase†

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ABSTRACT: The effects of hydrostatic pressure on three different preparations of mitochondrial H^+-ATPase were investigated by studies of the hydrolytic activity, of the spectral shift and quantum yield of the intrinsic protein fluorescence, and of filtration chromatography. Both membrane-bound and detergent-solubilized forms of the mitochondrial F_0-F_1 complex were reversibly inactivated in the pressure range of 600-1800 bar, whereas with soluble F_1-ATPase the inactivation was irreversible. Pressure inactivation of soluble F_1-ATPase was facilitated by decreasing the protein concentration, indicating that dissociation is an important factor. In the presence of 30% glycerol, soluble F_1-ATPase becomes inactivated by pressure in a reversible fashion, recovering the original activity. ATPase activity measured in an aqueous medium returns to the original values when incubated under high pressure in a glycerol-containing medium without substrate and is even enhanced when Mg-ATP is present. ATP hydrolysis returns to 80% of its original value in the case of the F_0-F_1 complex. Fluorescence studies under pressure revealed a red shift in the spectral distribution of the emission of tyrosine fluorescence of soluble F_1-ATPase. A decrease in the quantum yield of intrinsic fluorescence was also observed upon subjection to pressure. The fluorescence intensity decreased monotonically as a function of pressure when the sample was in an aqueous medium, whereas it presented a biphasic behavior in a 30% glycerol medium. Gel filtration studies demonstrated that the hydrodynamic properties of the F_1-ATPase are preserved if the enzyme is subjected to pressure in the presence of glycerol but they are modified when the same procedure is performed in an aqueous medium. It can be concluded that pressure dissociation of soluble F_1-ATPase is followed by reassociation to an inactive enzyme with altered hydrodynamic radius when the pressure is withdrawn. Protection against this irreversible effect by binding to the F_0 membrane component points directly to the importance of the coupling between membrane attachment and correct assembly of the F_1-ATPase.

Mitochondrial ATP synthase is the terminal enzyme in oxidative phosphorylation and catalyzes the synthesis of ATP with energy derived from electrochemical H^+ gradients. It is composed of a membrane sector, F_0, that permits the transport of protons generated by the respiratory chain to a soluble factor known as F_1 (Amzel & Pedersen, 1983; Senior & Wise, 1983). The F_1 moiety is composed of five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Amzel & Pedersen, 1983) and catalyzes ATP hydrolysis. Kinetic studies of the F_1-ATPase[1] have demonstrated that the three catalytic sites of the enzyme behave cooperatively (Gresser et al., 1982; Boyer et al., 1982; O'Neal & Boyer, 1984). These sites are ap-

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[1] Abbreviations: ATPase, adenosinetriphosphatase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SMP, submitochondrial particles.

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parently located in β subunits. The F_1-ATPase also contains low molecular weight subunits that are essential for catalysis. To date, few studies have been performed to ascertain subunit interactions that could regulate catalysis. In this study, we use hydrostatic pressure to alter subunit interactions and to examine the influence of environmental factors on subunit reassembling of ATP synthase. The dissociation of oligomeric proteins by pressure provides a method of inducing their reversible dissociation at concentrations at which this process can be readily followed by several spectroscopic techniques. The dissociation of oligomeric proteins by pressure seems to be a general phenomenon (Heremans, 1982; Weber & Drickamer, 1983; Weber, 1987). As initially shown by Penniston (1971), hydrostatic pressure tends to inhibit the enzymic activity of oligomeric complexes and to increase the activity of monomeric proteins. He found that F_1-ATPase was inhibited by pressure. Most of the studies of pressure-induced dissociation of proteins have employed optical methods. Light scattering has been useful when large aggregates are dissociated into many small particles (Payens & Heremans, 1969; Engelborghs et al., 1976), and fluorescence polarization and energy distribution of the fluorescence emission have been used to study the pressure dissociation of small oligomers, mostly dimers and tetramers (Paladini & Weber, 1981a; King & Weber, 1986a; Silva et al., 1986; Verjovski-Almeida et al., 1986; Royer et al., 1986). Polyacrylamide gel electrophoresis of oligomeric proteins under pressure has also been utilized to study the dissociating effect of pressure (Paladini et al., 1987).

Although pressure effects on complex oligomeric proteins are more difficult to interpret in detail, it seems to be the only tool that can provide information on protein–protein interactions with minimal direct effects on the tertiary structure of the proteins (Weber & Drickamer, 1983). The effects of pressure on a complex aggregated system, the brome mosaic virus, were recently described by us (Silva & Weber, 1987).

The present paper describes the effects of hydrostatic pressure (1–2400 bar) on three different preparations of the mitochondrial ATP synthase: submitochondrial particles, detergent-solubilized F_0–F_1 complex, and soluble F_1-ATPase. Pressure effects were followed by the decrease in the ATPase activity, changes of the spectral distribution and quantum yield of fluorescence, and changes of the hydrodynamic properties. The degree of recovery of enzymic activity and changes in the hydrodynamic behavior after decompression were investigated.

Materials and Methods

Chemicals. Pyruvate kinase from pig heart (400 units/mg), L-lactic dehydrogenase type II from rabbit muscle (940 units/mg), and rotenone were purchased from Sigma Chemical Co. (St. Louis, MO). FCCP was purchased from E. I. du Pont de Nemours and Co. All other reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system to 18-Mohm resistance.

Protein Preparations and Enzymic Activity. Heavy beef heart mitochondria and submitochondrial particles (SMP) were prepared essentially as described by Low and Vallin (1963); SMP were stored in liquid nitrogen until use. F_1-ATPase and crude F_0–F_1 complex solubilized by Triton X-100 were prepared according to reported procedures (Knowles & Penfsky, 1972; Tuena de Gomez-Puyou & Gomez-Puyou, 1977; Vasquez-Laslop & Dreyfus, 1986).

The measurements of ATPase activity under pressure following the oxidation of NADH (Pullman et al., 1960) were performed in a Durrum spectrophotometer adapted to contain the high-pressure bomb. It has been shown that pressure has no effect on the coupling enzymes in the pressure range of 1–2500 bar (Chong et al., 1985). A Varian (Cary 118) spectrophotometer was utilized for routine determinations of ATPase activity at atmospheric pressure.

Protein determinations were performed by the method of Lowry et al. (1951).

Fluorescence Studies under Pressure. The high-pressure bomb has been described by Paladini and Weber (1981a,b). The bomb is placed on a base that allows use of either 180 or 90° light paths. Therefore, ATPase activity or fluorescence can be measured directly through quartz windows in the bomb as described earlier (Paladini & Weber, 1981a,b; Chong et al., 1985; Silva et al., 1986). Fluorescence spectra were recorded on a ISS 200 spectrofluorometer (Champaign, IL). Fluorescence spectra at pressure p were quantitated by the specification of the center of spectral mass (υ_p)

$$\upsilon_p = \sum \upsilon_i F_i / \sum F_i \quad (1)$$

where F_i stands for the fluorescence emitted at wavenumber υ_i and the summation is carried out over the range of appreciable values of F. The importance of characterizing the spectral displacements by the mean wavenumber of the emission (eq 1) rather than by changes in maximum emission should be emphasized. The center of mass is an integral measure that can reach a precision of ± 10 cm^{-1}. In a heterogeneous system, such as that comprising the intrinsic fluorophores of a protein molecule, considerable displacements of the center of mass can take place with small or imperceptible changes in the maximum of emission.

Size Exclusion High-Performance Liquid Chromatography. This was performed in a prepacked SynChropak GPC300 column of 250 × 4.6 mm i.d., obtained from SynChrom, Inc. (Linden, IN). Typically, a flow rate of 0.3 mL/min was used. Elution of the sample was monitored by absorption or fluorescence. The void volume (V_0) of the column was measured with λ phage DNA and the total volume (V_t) with ADP. Calibration of the column was performed with proteins of different sizes. The partition coefficient K_d was calculated from the relation $K_d = (V_E - V_0)/(V_t - V_0)$, where V_E is the elution volume of the protein.

Results

Effects of Pressure on ATPase Activity. Membrane-bound, detergent-treated, and soluble ATPase preparations were subjected to hydrostatic pressure in the range of 1–2400 bar. Figure 1 shows the time course of ATP hydrolysis at atmospheric pressure, at pressures that almost completely abolish the activity of each preparation, and after the return to atmospheric pressure. ATPase activity is completely recovered after pressure is released in the case of submitochondrial particles (Figure 1A) or soluble F_0–F_1 complex (Figure 1B). However, hydrolytic activity is irreversibly lost in the case of F_1-ATPase (Figure 1C) after return to atmospheric pressure. As shown in Figure 1, both the decrease in activity induced by pressure and the reactivation after return to atmospheric pressure were faster than the time resolution of the method. In the case of submitochondrial particles and of solubilized F_0–F_1 complex, the recovery of full activity after release of pressure occurred even after incubation under 1.7 kbar for 1 h (data not shown).

The irreversible loss of F_1-ATPase activity caused by high pressure can be overcome by the presence of 30% glycerol. At atmospheric pressure, after an initial lag of ~15 min the activity of F_1-ATPase in the presence of glycerol reaches its maximal value, which is ~30% of the activity in aqueous medium (Figure 2A). This lag behavior is the objective of

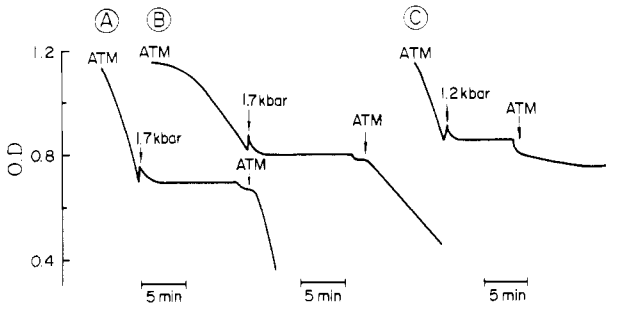

FIGURE 1: Effect of pressure on ATPase activity. (A) Submitochondrial particles (0.02 mg/mL); (B) Triton X-100 solubilized F_0–F_1 complex (0.06 mg/mL); (C) soluble F_1-ATPase (0.0053 mg/mL). The reaction media contained 0.025 M Tris-acetate, pH 8.0, 0.25 M sucrose, 0.03 M potassium acetate, 78 units/mL of pyruvate kinase and lactic dehydrogenase, 4 mM phosphoenolpyruvate, 3 mM $MgSO_4$, 3 mM ATP, 1 mM NADH, and 2 μM FCCP/rotenone in the case of submitochondrial particles. The reaction was started by addition of the different ATPase preparations. ATP hydrolysis was followed spectrophotometrically through the oxidation of NADH at 340 nm. The temperature was 25 °C.

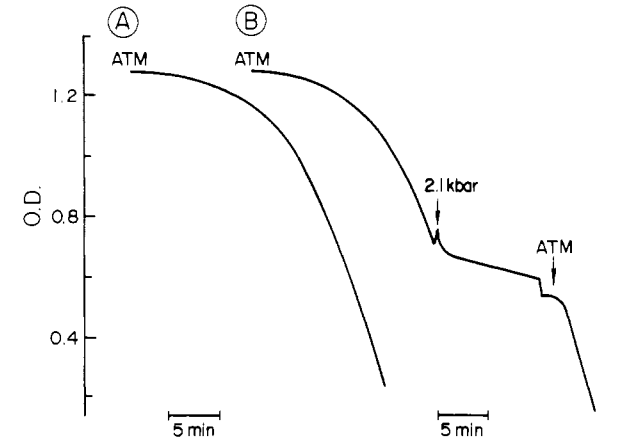

FIGURE 2: Effect of glycerol on the pressure-induced inactivation of ATP hydrolysis in F_1-ATPase. Reaction media as described in Figure 1 but containing 30% glycerol. (A) ATPase activity of F_1-ATPase (0.015 mg/mL) at atmospheric pressure; (B) ATPase activity at atmospheric pressure, at 2.1 kbar, and after return to atmospheric pressure.

another paper (manuscript in preparation). Figure 2B shows that ATPase activity in the presence of glycerol is inhibited at 2.1 kbar, but is completely recovered when pressure is released, in contrast to the irreversibility found with F_1-ATPase in aqueous medium (Figure 1C). Table I shows the absolute values of hydrolytic activity of soluble F_1-ATPase measured in aqueous medium in atmospheric pressure before and after subjection to a pressure of 2.2 kbar for 10 min. It is shown that ATP hydrolysis is almost completely abolished if compression is carried out in an aqueous medium. This condition differs from that in Figure 1C because a higher protein concentration was used and no substrate was present. Oppositely, if F_1-ATPase is subjected to the same treatment in a medium containing 30% glycerol, the ATPase activity measured after dilution into aqueous medium is preserved. If in addition to glycerol the substrate Mg-ATP is present during the pressure incubation, the activity after pressure treatment is higher than that of the sample kept at atmospheric pressure.

Pressure Inactivation Curves. In order to ascertain whether the loss of activity induced by pressure is due to tertiary conformational changes or dissociation, a study of the dependence upon protein concentration was performed. A dissociation process must occur more readily as the total protein concentration is decreased, whereas a tertiary conformational

Table I: Pressure Effects on ATP Hydrolysis of F_1-ATPase

additions to incubation medium[a]	ATP hydrolysis[b] before pressure	ATP hydrolysis[b] after pressure
(a) none	29.0	0.035
(b) 30% glycerol medium without Mg-ATP	32.0	35.0
(c) 30% glycerol + Mg-ATP	41.1	63.0

[a] F_1-ATPase (18 μg/mL) was subjected to a pressure of 2.5 kbar for 10 min in an incubation medium containing 0.025 M Tris-acetate, pH 8.0, 0.25 M sucrose, and 0.03 M potassium acetate, at 25 °C (a). In (b), the medium also contained 30% glycerol; in (c), it contained glycerol plus 3 mM ATP, 3 mM $MgSO_4$, and regenerating system as described in Figure 1. [b] ATPase activity (expressed in μmol min^{-1} mg^{-1}) was measured spectrophotometrically at atmospheric pressure after transferring an aliquot of the incubation medium to 1 mL of a reaction mixture containing an ATP-regenerating system (200 μM NADH and 14 units each of pyruvate kinase and lactate dehydrogenase).

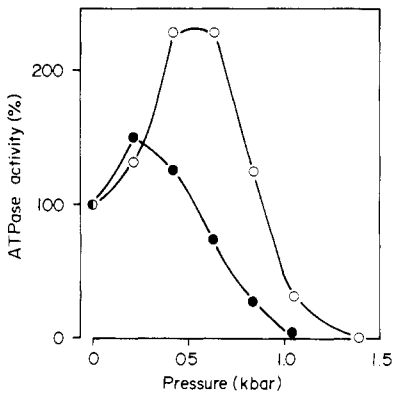

FIGURE 3: Pressure inactivation of F_1-ATPase at two different protein concentrations. ATPase activity vs pressure at two concentrations: 8 μg/mL F_1-ATPase (○) and 2 μg/mL F_1-ATPase (●). Other conditions as in Figure 1.

change, a typical first-order reaction, is concentration independent [for reviews see Weber and Drickamer (1983) and Weber (1987)]. Figure 3 shows the effects of pressure on the ATPase activity of soluble F_1 sector at two different protein concentrations. For both concentrations, the ATPase activity increases in a low-pressure range, followed by inactivation at higher pressures. The inactivation occurs at lower pressures when the protein concentration is 2 μg/mL than when it is 8 μg/mL. Thus, it appears that dissociation is the underlying process for the pressure-induced inactivation. The increase of activity in low pressures may be explained by a negative volume change of activation of ATPase activity.

The effect of pressure on ATP hydrolysis by submitochondrial particles is shown in Figure 4A. ATPase activity decreases as the pressure is increased, attaining 50% of its initial value at a pressure of ~850 bars. A marked hysteresis is observed: The hydrolytic activity measured at each pressure is systematically higher during compression than during the return to atmospheric pressure. A concentration dependence study was not undertaken with the submitochondrial particles because the F_0–F_1 component is not freely dispersed in solution but is attached to the membrane. Hysteretic behavior is also observed when Triton-solubilized F_0–F_1 complex is subjected to pressure (Figure 4B). The hysteresis found with both the membrane-attached and solubilized forms of F_0–F_1 complex may be indicative of a decrease in affinity between subunits upon dissociation, a phenomenon previously reported for homodimers (Silva et al., 1986) and homotetramers (King & Weber, 1986a). Interestingly, the hysteretic behavior seen with the membrane in detergent-solubilized preparations is inverted in the presence of glycerol, i.e., the ATPase activities are higher

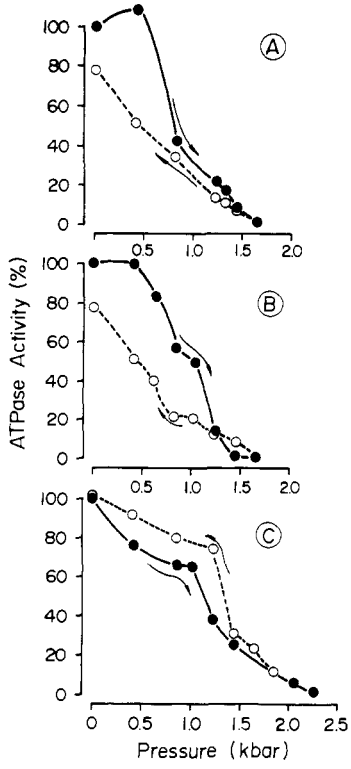

FIGURE 4: Hysteresis of the pressure-inactivation curves. (A) Submitochondrial particles (0.02 mg/mL); (B) Triton X-100 solubilized F_0–F_1 ATPase (0.063 mg/mL); (C) soluble F_1-ATPase in the presence of 30% glycerol (0.005 mg/mL). ATPase activity measured in the pressure bomb was plotted with the starting activity at atmospheric pressure as 100%. Other conditions as described in Figure 1.

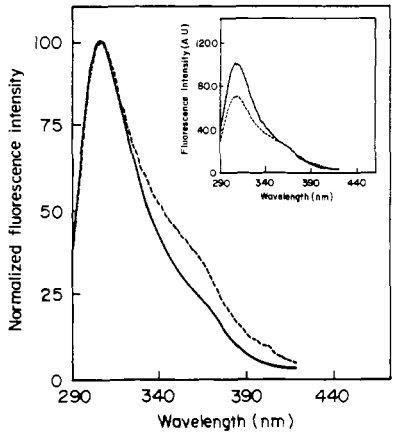

FIGURE 5: Effect of pressure on the intrinsic fluorescence emission of F_1-ATPase. Normalized intrinsic fluorescence spectra of 180 μg/mL F_1-ATPase (0.5 μM) at atmospheric pressure (—) and at 2.4 kbar (---), excited at 280 nm, in a medium containing 0.025 M Tris-acetate, pH 8.0, 0.25 M sucrose, and 0.03 M potassium acetate. Inset: Nonnormalized emission spectra of F_1-ATPase at atmospheric pressure (—) and at 2.4 kbar (---). The excitation wavelength was 280 nm.

for the decompression curve than for the compression curve (Figure 4C).

Pressure Effects on the Intrinsic Fluorescence of F_1-ATPase. The spectral distribution of the intrinsic fluorescence of F_1-ATPase at different pressures was examined in order to ascertain whether changes in the environment of aromatic residues occurred in parallel with inactivation. Figure 5 illustrates the emission spectra of a solution of F_1-ATPase in aqueous medium at atmospheric pressure and at high pressure (2.4 kbar). The fluorescence emission is characteristic of tyrosine groups with virtually no contribution from tryptophan,

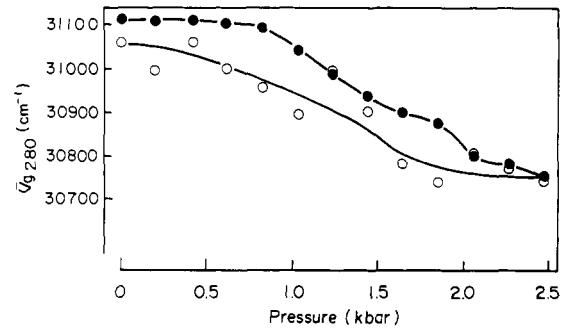

FIGURE 6: Concentration dependence of the red shift of the emission of F_1-ATPase. Plot of center of spectral mass (v_p) vs pressure at two concentrations, 0.5 μM (180 μg/mL) F_1-ATPase (●) and 0.05 μM (18 μg/mL) F_1-ATPase (○). The center of mass v_p was calculated as indicated in Materials and Methods. Molar concentrations of F_1-ATPase were calculated by using a molecular weight of 360 000. Other conditions as in Figure 5.

in agreement with the described lack of this residue in the whole protein complex (Amzel & Pedersen, 1983). The excitation spectrum is also characteristic of tyrosine groups (data not shown). The emission spectrum of F_1-ATPase under pressure undergoes a red shift (Figure 5) and a decrease in the fluorescence quantum yield (inset of Figure 5). The more likely explanation for the red shift is that at high pressure one or more intrinsic fluorophores become exposed to a medium of increased polarity, presumably water. The difference between the average wavenumbers of the high-pressure and atmospheric pressure spectra was 300 cm^{-1}. This change in the center of mass of intrinsic fluorescence is of the same magnitude of those observed in other dissociating systems (Silva et al., 1986; Royer et al., 1987; Silva & Weber, 1987). The difference of electronic distribution between the excited and ground states in tyrosine is smaller than in tryptophan, i.e., tyrosine groups are less sensitive to environmental polarity. Therefore, the changes in interaction of tyrosine groups with its surroundings do not promote as larger displacements of v as those found with tryptophans (Silva et al., 1986; Silva & Weber, 1987).

The fluorescence changes shown in Figure 5 were measured at a pressure that completely inactivates the enzyme, suggesting that high pressure is provoking substantial structural changes, probably in the quaternary structure of the protein. Figure 6 shows that the center of spectral mass decreased monotonically as a function of pressure and leveled off at ~2.2 kbar. The shift in center of mass was more pronounced at a lower protein concentration. The pressure at which the half-maximal effect was observed ($p_{1/2}$) was ~200 bar smaller for a 10-fold diluted sample. This difference in $p_{1/2}$ is smaller than that observed for the effect of pressure on ATP hydrolysis in Figure 3. This seems to indicate that the change in microenvironment of the fluorophore is not only brought about by dissociation but probably has the contribution of conformational changes that occur in the dissociated products. Thus, we cannot attribute fixed values of v for the aggregated and dissociated forms.

The spectral shift was smaller in the presence of glycerol (<100 cm^{-1}; data not shown), indicating that much of the change observed in water may arise from the irreversible changes that follow dissociation when glycerol is not present. Figure 7 shows that the quantum yield of tyrosine fluorescence decreases monotonically as a function of pressure when F_1-ATPase is in an aqueous medium, whereas a biphasic behavior is noted in a medium containing 30% glycerol.

Structural Properties. The hydrodynamic properties of soluble F_1-ATPase after treatment with pressure were inves-

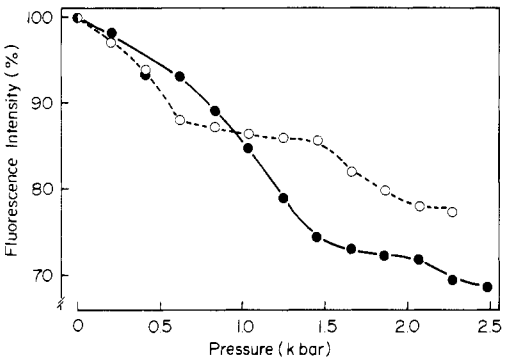

FIGURE 7: Pressure effects on the fluorescence intensity of F_1-ATPase at the emission of 306 nm. F_1-ATPase (0.5 μM) was subjected to pressure in a medium identical with that of Figure 5 in the absence (●) and in the presence (○) of 30% glycerol. The excitation wavelength was 280 nm.

tigated by filtration high-performance liquid chromatography (HPLC). The sizing HPLC column was calibrated with globular proteins (not shown). The elution position of F_1-ATPase detected either by its absorbance at 254 nm or by its intrinsic fluorescence corresponded to a particle of 57 ± 0.6 Å (Figure 8A). Figure 8B shows the elution of F_1-ATPase after incubation in an aqueous medium for 10 min at 2.2 kbar. Its hydrodynamic radius is clearly higher (63 ± 0.7 Å) than that obtained for the sample that was incubated at atmospheric pressure. When the same pressure treatment was conducted in the presence of 30% glycerol, there was no detectable change in the elution position of the pressurized sample in comparison to the control sample incubated at atmospheric pressure (Figure 8C,D). The increase in the hydrodynamic size of F_1-ATPase subjected to pressure in the absence of glycerol indicates that reassociation after release of pressure occurs anomalously and is probably the underlying molecular cause of the irreversible inactivation.

Discussion and Conclusions

The main goal of this study was to use hydrostatic pressure to perturb the protein–protein interactions of the mitochondrial ATP synthase. We demonstrate that three different preparations of F_1-ATPase are inactivated by pressure in the range of 500–2000 bar, the same range in which, with other proteins, the dissociation of dimers (Paladini & Weber, 1981; Silva et al., 1986; Ruan & Weber, 1987), tetramers (King & Weber, 1986; Royer et al., 1986), and larger aggregates (Payens & Heremans, 1969; Engelborghs et al., 1976; Silva & Weber, 1987) has been observed. This study furnishes evidence that F_1-ATPase inactivation is caused by protein dissociation. From pressure studies on single polypeptides it has been concluded that the tertiary conformation of proteins is not altered significantly in the pressure range from atmospheric to 4 kbar [for a review see Weber and Drickamer (1983)]. More direct information about the source of pressure-induced inactivation of F_1-ATPase was obtained by experiments at low and high protein concentrations. Both the inactivation and the fluorescence changes in F_1-ATPase induced by pressure were protein concentration dependent (Figures 3 and 6).

The pressure dissociation data permit the calculation of thermodynamic parameters related to the subunit association reaction, namely, the dissociation constant at atmospheric pressure and the standard volume change of association (Paladini & Weber, 1981; Weber & Drickamer, 1983; Silva et al., 1986). To calculate these parameters it is necessary to know the order of the dissociation reaction. On the assumption that F_1-ATPase is fully active only when the whole structure

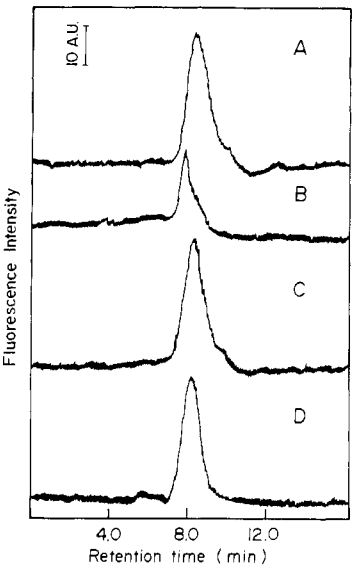

FIGURE 8: Size exclusion HPLC of F_1-ATPase after pressure treatment. The intrinsic fluorescence (excitation at 280 nm, emission at 315 nm) elution of F_1-ATPase was monitored: (A) 50 μL of 0.5 μM F_1-ATPase in aqueous buffer incubated at atmospheric pressure was injected; (B) 0.5 μM F_1-ATPase was subjected to 2.4 kbar for 10 min, and 50 μL of the samples was injected into the GPC 500 column after release of pressure; (C) as in (A) except that 30% glycerol was present; (D) as in (B) except that the sample was subjected to pressure in the presence of 30% glycerol. The flow rate was 0.3 mL/min and the elution buffer was 0.05 M Tris, pH 7.0, 0.25 M sucrose, and 0.2 M sodium acetate.

is present or not covalently modified, a second order can be assigned for the dissociation when it is determined by ATP hydrolysis activity. This assumption is supported by two lines of reported findings. The first line shows an almost total inactivation when only one β catalytic subunit is modified by antibiotic binding or covalent derivatization (Chang & Penefsky, 1973; Ferguson et al., 1975; Verschoor et al., 1977; Kohlbrenner & Cross, 1978; Lunardi et al., 1979). The second line of evidence is the positive catalytic cooperativity between the three sites, which has been demonstrated by the increase of rate of release of ADP + P_i by binding of ATP (Kayalar et al., 1977; Hackney & Boyer, 1978; Choate et al., 1977; Cross, 1981).

The dissociation constant at pressure p (K_p) and at atmospheric pressure (K_{atm}) are related by

$$K_p = K_{atm} \exp(p\, dV^\circ/RT) \quad (2)$$

where p is pressure (in bars) and dV° is the standard volume change upon association (in milliliters per mole). If we introduce the degree of dissociation at pressure p, a_p, we can write eq 2 for the case of a second-order dissociation reaction in the form

$$\ln [a_p^2/(1 - a_p)] = p\, dV^\circ/RT + \ln (K_{atm}/4C) \quad (3)$$

where $K_p = 4Ca_p^2/(1 - a_p)$, with C as the concentration of the protein. We utilized this expression to derive the dissociation constant and the volume change of the pressure inactivation of soluble F_1-ATPase in the presence of glycerol, where the reaction is fully reversible and the system is soluble. A molecular weight of 360 000 was used for the calculation of the molar concentration of F_1-ATPase. A plot of $\ln [a_p^2/(1 - a_p)]$ against the pressure allows one to calculate the volume change (dV°) from the slope and the dissociation constant from the intercept. The dV° and K_d derived from the pressure inactivation curve for F_1-ATPase in the presence of glycerol were, respectively, 76 mL/mol and 7.1×10^{-9} M. This value

of volume change is of the same order of magnitude as that observed for protein dimers [60 mL/mol for yeast enolase (Paladini & Weber, 1981); 150 mL mol^{-1} for tryptophan synthase (Silva et al., 1986)] and tetramers [240 mL mol^{-1} for porcine lactate dehydrogenase (King & Weber, 1986)].

The irreversible pressure inactivation of F_1-ATPase as compared to the return to original activity in the case of the membrane or solubilized F_0–F_1 complexes brings about insights concerning the assembly of the ATP synthase system. The hydrodynamic properties of reassociated F_1-ATPase that was dissociated by pressure were studied by filtration chromatography (Figure 8). These studies provided a structural basis for the irreversible loss of activity of F_1-ATPase. Pressure-activated F_1-ATPase has an increased Stokes' radius. On the other hand, the hydrodynamic properties of the F_1-ATPase were preserved when the enzyme was subjected to pressure in the presence of glycerol. The inactive enzyme with altered hydrodynamic properties after pressure dissociation in aqueous buffer can be explained by the wrong association of the subunits when the pressure is released. It should be emphasized that the increase of $\sim$10% of the Stokes radius is more likely to be explained by a different quaternary structure organization rather than a gross denaturation. Either the binding of the F_0 membrane component or the presence of glycerol protects against the irreversible effect, probably by preventing conformational changes in the dissociated form that are responsible for the association to the "defective form". We cannot provide a definitive explanation for the protection against the irreversible pressure inactivation elicited by glycerol. It may simulate the environment of the membrane plus F_0 component.

The reassociation to a defective form seems to have the same nature of the irreversible loss of ability to form virus capsids after a cycle of dissociation–association of brome mosaic virus (Silva & Weber, 1987). These phenomena are similar to those found with simple oligomeric proteins, in which a loss of free energy of association of the subunits has been observed when these became separated following changes in concentration (Xu & Weber, 1982), pressure (King & Weber, 1986a; Silva et al., 1986), and temperature (King & Weber, 1986b). We have concluded that the observed effects result from slow changes in the conformation of the dissociated subunits that take place when they become separated from each other, regardless of the cause of separation. The loss of contacts between the subunits results in changes in their conformation so that an aggregate of diminished subunit affinity is formed when the particles eventually reassociate. This has been named a conformational drift of the separated subunits.

The hysteresis of the pressure inactivation curves observed for membrane and solubilized F_0–F_1 complexes indicates that a small loss of affinity among subunits has occurred at high pressures where the subunits are dissociated. This is consistent with the hypothesis of a conformational drift. In the case of F_1-ATPase in aqueous medium, this effect is more drastic and results in complete irreversible loss of activity. Comparison of the effects of pressure upon dimers (Paladini & Weber, 1981; Silva et al., 1986) and tetramers (King & Weber, 1986; Royer et al., 1986) with those on complex aggregates such as F_1-ATPase (this paper) and viruses (Silva & Weber, 1987) permits the conclusion that qualitatively the conformational drift effects are more remarkable in the latter aggregates.

F_1-ATPase is also inactivated when incubated in the cold (Penefsky & Warner, 1965; Bruni et al., 1977). Glycerol also protects against cold inactivation (Penefsky & Warner, 1965; Dreyfus & de Meis, 1988) as well as against pressure inactivation, suggesting a common underlying mechanism for the two phenomena. Inactivation induced by cold incubation similar to that caused by pressure has been demonstrated for lactate dehydrogenase (King & Weber, 1986; Silva & Weber, 1986).

In vivo assembly of the mitochondrial ATP synthase is a challenging problem. Our data suggest that assembly of an active soluble F_1-ATPase is unlikely. Therefore, the association of the five different types of subunit into the final constitution $\alpha_3\beta_3\gamma\delta\epsilon$ is not random and probably occurs at some fixed site. Successful reconstitution of F_1-ATPase was obtained with the *Escherichia coli* but not with the mitochondrial enzyme [for reviews see Senior and Wise (1983) and Senior (1985)]. In view of the results obtained in our study, the unsuccessful reconstitution of mitochondrial F_1-ATPase may be explained by conformational drift. This conformational change that follows the separation of the subunits is probably less dramatic in the *E. coli* F_1-ATPase. Pressure studies on the *E. coli* enzyme should provide an interesting subject for further studies.

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Registry No. ATPase, 9000-83-3; ATP, 56-65-5; ATP synthase, 37205-63-3; glycerol, 56-81-5.

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Pyridoxal 5′-Phosphate Mediated Inactivation of *Escherichia coli* DNA Polymerase I: Identification of Lysine-635 as an Essential Residue for the Processive Mode of DNA Synthesis†

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ABSTRACT: Inactivation of *Escherichia coli* DNA polymerase I by pyridoxal 5′-phosphate treatment results from its reactivity at multiple lysine residues. One of these residues, lysine-758, has been shown to be located at the substrate binding site in DNA polymerase I [Basu, A., & Modak, M. J. (1987) *Biochemistry 26*, 1704-1709]. We now demonstrate that lysine-635 is another important target of pyridoxylation; modification of this site results in decreased rates of DNA synthesis. Addition of template-primer with or without substrate deoxynucleoside triphosphate protects lysine-635 from pyridoxylation. Analysis of the initiation versus elongation phase of DNA synthesis by lysine-635-modified enzyme revealed that elongation of the DNA chain is severely affected by the lysine-635 modification. We therefore conclude that this lysine residue plays an important role in the processive mode of DNA synthesis by *E. coli* DNA polymerase I.

Enzymatic synthesis of DNA is a complex process since it requires an orderly interaction of multiple components. Resolution of the crystal structure of the Klenow fragment of *Escherichia coli* DNA polymerase I (Ollis et al., 1985) has provided the first glimpse of the three-dimensional architecture of this unique protein which carries domains responsible for template-primer and substrate deoxynucleoside triphosphate binding. With the help of site-specific reagents, we have shown that a simple process of substrate binding involves multiple amino acid residues which are situated quite apart in the primary sequence but are actually in close atomic proximity in the three-dimensional structure. For example, with pyridoxal 5′-phosphate (PLP)[1] as a substrate binding site directed labeling reagent (Modak, 1976; Hazra et al., 1984), we demonstrated involvement of lysine-758 (Basu & Modak, 1987) in the process of substrate binding. Yet, with the technique

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[1] Abbreviations: pol I, *Escherichia coli* DNA polymerase I; PLP, pyridoxal 5′-phosphate; dNTP, deoxynucleoside triphosphate; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; BSA, bovine serum albumin; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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