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# Dissociation of Purified Erythrocyte $Ca^{2+}$-ATPase by Hydrostatic Pressure*

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**Subunit interactions in the $Ca^{2+}$-ATPase from erythrocyte plasma membranes were investigated through a combination of fluorescence spectroscopy and high-pressure techniques. Application of hydrostatic pressure in the range of 1 bar to 2.4 kbar promoted full dissociation of the ATPase, as revealed by spectral shifts of the intrinsic fluorescence emission and by changes in the fluorescence polarization of dansyl-conjugated ATPase. Pressure dissociation of the ATPase displayed a dependence on protein concentration compatible with dissociation of a dimer. Calculated from pressure-dissociation curves, the standard volume change $dV^0$ for the association of subunits was 43–50 ml/mol and $K_0$, the dissociation constant at atmospheric pressure, was 6–9 × $10^{-8}$ M. Addition of $Ca^{2+}$ stabilized the dimeric ATPase structure against pressure dissociation, whereas addition of vanadate facilitated dissociation by pressure. These results suggest that intersubunit interactions depend on the equilibrium between the two major conformational states $E_1$ and $E_2$ of the ATPase. Addition of calmodulin in the presence of $Ca^{2+}$ had no additional effect when compared to that observed in the presence of $Ca^{2+}$ alone. This finding is interpreted in terms of the mechanism of calmodulin activation of ATPase catalysis.**

The erythrocyte plasma membrane $Ca^{2+}$-ATPase is responsible for maintaining submicromolar $Ca^{2+}$ concentrations in the cytoplasm of the red cell (Lew *et al.*, 1982; Carafoli, 1987; Schatzmann, 1989). In analogy with sarcoplasmic reticulum ATPase, the erythrocyte $Ca^{2+}$-ATPase undergoes isomerization between two major conformational states, $E_1$ and $E_2$, depending on whether $Ca^{2+}$ is bound or not to high-affinity sites on the enzyme (Kosk-Kosicka and Inesi, 1985; Krebs *et al.*, 1987). CaM[1] binds to the erythrocyte ATPase in the presence of $Ca^{2+}$ and regulates both ATP hydrolysis and $Ca^{2+}$ pumping activities (Gopinath and Vincenzi, 1977; Niggli *et al.*, 1982). Spectroscopic studies have revealed that a distinct conformational state, different from the $Ca^{2+}$-activated state, is associated with the enzyme activated by CaM[2] (Wrzosek *et al.*, 1989). Further studies have shown that not only CaM but also acidic phospholipids, unsaturated fatty acids, limited proteolysis, and protein kinase-dependent phosphorylation are able to promote full activation of the enzyme (Niggli *et al.*, 1981; Neyses *et al.*, 1985; Smallwood *et al.*, 1988). It has recently been established by Kosk-Kosicka and co-workers that self-association of ATPase monomers in an oligomeric structure is an important feature in the activation of the enzyme (Kosk-Kosicka and Bzdega, 1988, 1990; Kosk-Kosicka *et al.*, 1989).

In this study, the self-association of the erythrocyte ATPase was directly investigated by using hydrostatic pressure as an external variable to perturb the equilibrium of subunit association. Full dissociation of the erythrocyte $Ca^{2+}$-ATPase was achieved using hydrostatic pressure. Pressure-induced dissociation was monitored by measurements of both intrinsic fluorescence and fluorescence polarization of ATPase covalently labeled with 2,5-dansyl chloride. The combination of fluorescence spectroscopy and high-pressure techniques has been used to investigate the dissociation of several proteins (for a review see Weber, 1987), including the dissociation of sarcoplasmic reticulum $Ca^{2+}$-ATPase in detergent solution (Verjovski-Almeida *et al.*, 1986).

Our results show that the dissociation of the oligomeric erythrocyte $Ca^{2+}$-ATPase corresponds to a dimer-monomer transition. Addition of $Ca^{2+}$ increases stability of the dimer toward pressure. By contrast, removal of $Ca^{2+}$ or addition of orthovanadate, an analog of inorganic phosphate, favors monomerization. This result suggests that the activation state of the ATPase and the tendency of subunits to associate may be closely related. CaM, in the presence of $Ca^{2+}$, had no additional effect compared to that observed with $Ca^{2+}$ alone.

## MATERIALS AND METHODS

*Reagents*—Nonionic detergent $C_{12}E_8$ was obtained from Nikkol (Tokyo, Japan). Bovine brain calmodulin, egg yolk phosphatidylcholine, and sodium orthovanadate were from Sigma. Calmodulin-Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden), and 2,5-dansyl chloride was from Molecular Probes (Eugene, OR). All other reagents were of the highest purity available.

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[1] The abbreviations used are: CaM, calmodulin; $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; 2,5-dansyl chloride, 2-dimethylaminonaphthalene-5-sulfonyl chloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[2] T. Coelho-Sampaio and S. T. Ferreira, submitted for publication.

*Enzyme Preparation*—$Ca^{2+}$-ATPase was affinity-purified from human erythrocyte ghosts as described by Benaim *et al.* (1984), except that $C_{12}E_8$ was used instead of Triton X-100, as previously described by Kosk-Kosicka and Inesi (1985). The substitution of the detergent was necessary to enable fluorescence measurements. Samples were stored at −70 °C in buffer containing 20 mM Hepes, pH 7.4, 130 mM KCl, 3 mM $MgCl_2$, 50 μM $CaCl_2$, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mg/ml phosphatidylcholine, 0.5 mg/ml $C_{12}E_8$, and 5% (v/v) glycerol. Protein concentration was measured as described by Bensadoun and Weinstein (1976). A molecular weight of 140,000 was assumed for the monomeric ATPase. Free $Ca^{2+}$ was calculated using a computer program based on an iterative method that took into account the different species involved in the equilibrium between EDTA, $Ca^{2+}$, $Mg^{2+}$, $K^+$, and $H^+$, as originally described by Fabiato and Fabiato (1979), with the modifications introduced by Sorenson *et al.* (1986). Samples for fluorescence measurements were diluted to the desired protein concentrations in 130 mM KCl plus 4 mM EDTA and then purged with argon to prevent photooxidation.

*Dansyl Labeling of the ATPase*—Erythrocyte ghosts (400 mg of total protein) suspended in buffer containing 30 mM Hepes, pH 7.4, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, and 130 mM KCl were centrifuged at 17,000 × *g* for 50 min and resuspended in 80 ml of hypotonic medium (30 mM Hepes, pH 7.8, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$). 2,5-Dansyl (47 μmol) was dissolved in 0.8 ml of dimethylformamide immediately before use and was added to the leaky ghosts. Reaction was carried out at room temperature in the dark, with gentle stirring. After 60 min the pH was returned to 7.4 with diluted HCl and 130 mM KCl was added; the mixture was immediately solubilized with $C_{12}E_8$, and the ATPase was purified as described above. Labeling was confirmed by the observation of fluorescence resonance energy transfer from ATPase tryptophan residues to the probe (see Fig. 3, *inset*).

*Fluorescence Measurements*—Steady-state fluorescence measurements were performed at 22 °C on an ISS (Champaign, IL) GREG-PC spectrofluorometer. In dilution experiments, appropriate blanks were subtracted from the spectra at each protein concentration to correct for background interference, which was significant at low enzyme concentrations (~60% at 10 μg/ml). Excitation was at 275 nm, unless otherwise indicated. Spectral centers of mass (average emission wavenumber) were calculated as $\nu_{av} = \int \nu I(\nu) d\nu / \int I(\nu) d\nu$, where $\nu$ and $I(\nu)$ are wave number (in $cm^{-1}$) and fluorescence intensity at a given wave number, respectively. Experiments under pressure were performed using the pressure bomb described by Paladini and Weber (1981). Samples were kept for 10 min at each pressure before measurements. In the pressure range employed (1–2400 bar) no direct effects of pressure are observed in the tertiary structure of proteins (Heremans, 1982; Weber and Drickamer, 1983).

Fluorescence polarization measurements of dansyl-ATPase were performed at excitation and emission wavelengths of 340 and 415 nm, respectively. Polarization values measured in the high-pressure bomb were corrected for the birefringence of the quartz windows (Paladini and Weber, 1981) and for background fluorescence (~15%).

Lifetime measurements of dansyl-ATPase were performed at 10 different frequencies from 1 to 100 MHz on a multifrequency phase-modulation fluorometer. Excitation was provided by the 325-nm line of a Liconix HeCd laser previously modulated by a Pockels cell. Excitation was at magic angle configuration and emission was collected through a KV 418 filter. A reference sample of 1,4-bis[2-(phenyloxazolyl)]benzene in ethanol (lifetime = 1.32 ns) was used to minimize color errors due to photomultiplier response. The measured lifetime for dansyl-ATPase was 29.95 ns.

All results presented here are representative of at least three experiments using different ATPase preparations.

## RESULTS

*Effect of ATPase Dilution on the Fluorescence Emission*—Fig. 1 shows the centers of mass of ATPase fluorescence emission spectra at different ATPase concentrations. ATPase samples were diluted in KCl/EDTA buffer, keeping constant the ratio of protein/detergent/phospholipid concentrations (1:2:2, in milligrams). The center of mass was not affected by dilution from 250 to 80 μg/ml (Fig. 1). Further dilution down to 10 μg/ml promoted a red shift of 220 $cm^{-1}$ in the emission spectrum (Fig. 1). The red shift suggests an increase in the polarity of the medium to which fluorophores are exposed (Weber, 1987). This behavior has been reported to occur upon

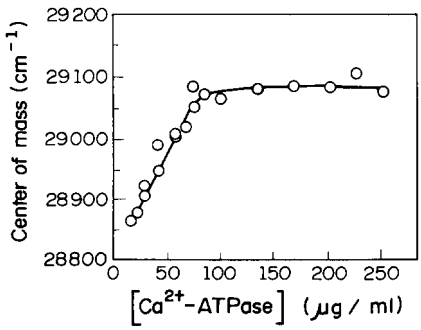


FIG. 1. **ATPase concentration dependence of intrinsic fluorescence emission.** ATPase samples were diluted to the indicated protein concentrations in medium containing 4 mM EDTA plus 130 mM KCl, keeping constant the ratio of protein:detergent:phospholipid concentrations (1:2:2, in mg). Prior to dilution, EDTA was added to the samples to maintain the EDTA concentration constant at 4 mM. Samples were purged with argon, and emission spectra (excitation at 275 nm) were acquired from 315 to 420 nm. Spectral centers of mass were calculated as described under "Materials and Methods," after subtraction of appropriate blanks. Errors in measurements of centers of mass were less than 10 $cm^{-1}$.

dissociation of several oligomeric proteins, as a consequence of the exposure of tryptophan residues previously buried in intersubunit interfaces to the aqueous medium (Thompson and Lakowicz, 1984; Weber, 1987). It is noteworthy that the effect of dilution on the spectral shift was not yet complete at 10 μg/ml, as indicated by the absence of a plateau at low protein concentrations (Fig. 1). This fact indicates that full dissociation of the ATPase was not achieved by dilution in the concentration range tested. Accurate measurements below 10 μg/ml were not possible due to significant contributions from background fluorescence (see "Materials and Methods").

*Effect of Pressure on the Fluorescence Emission of the ATPase*—Hydrostatic pressure has been widely used to study protein quaternary structure and has the advantage of circumventing the difficulty in performing measurements at the very low protein concentrations required for dissociating proteins by dilution (Weber, 1987). Fig. 2*A* shows a comparison between ATPase emission spectra acquired at atmospheric pressure and at 2.4 kbar. Application of pressure resulted in a red shift of the emission spectrum, similarly to what occurred upon dilution of the protein (Fig. 1). This result indicated that increased pressure promoted dissociation of the ATPase, as confirmed below. It is interesting to note, however, that the red shift observed at 30 μg/ml of ATPase (Fig. 2*A*) was about 100 $cm^{-1}$, which is smaller than that found for the dissociation of other oligomeric proteins as tryptophan sinthase, hexokinase, and glicerol-phosphate dehydrogenase reported to be between 600 and 1100 $cm^{-1}$ (Silva *et al.*, 1986; Ruan and Weber, 1988; Ruan and Weber, 1989). This fact could be explained if tryptophan residues are located on the surface of the ATPase oligomer instead of at the interface between subunits. For this reason, we measured the quenching of the intrinsic fluorescence of the ATPase by a hydrophilic quencher, acrylamide, in order to assess the accessibility of tryptophan residues at atmospheric pressure (Lakowicz, 1983). Fig. 2*B* shows a Lehrer plot (Lehrer, 1971) for acrylamide quenching data. From this type of plot we obtain $f_a$ (fraction of accessible tryptophan residues) as well as $K_a$ (quenching constant). The calculated values were 0.91 and 0.145 M, respectively. The conjunction of this very high quenching constant, similar to the Stern-Volmer constant found by Wrzosek and co-workers (1989), with an $f_a$ value near unity, indicates that tryptophan residues in the ATPase are probably on the surface of the protein and not in the

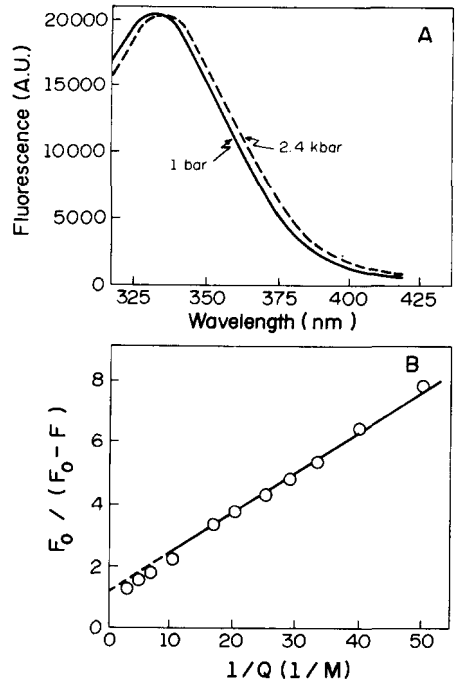


FIG. 2. *A*, effect of hydrostatic pressure on the ATPase fluorescence emission spectrum. ATPase was diluted to 30 μg/ml as described in the legend to Fig. 1. The *continuous line* shows the emission spectrum (excitation at 275 nm) of erythrocyte $Ca^{2+}$-ATPase at atmospheric pressure. The *dashed line* is the spectrum obtained at 2.4 kbar of pressure, normalized to the same intensity as the spectrum at 1 bar. *B*, acrylamide quenching of ATPase fluorescence emission. Acrylamide concentration was changed by addition of small volumes of freshly prepared 2 M acrylamide stock solution. ATPase fluorescence intensity (excitation = 275 nm; emission = 335 nm) was measured after each addition of acrylamide. Data have been corrected for dilution and are plotted according to the Lehrer equation as follows.

$$\frac{F_o}{F_o - F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a}$$

where $F_o$ is the fluorescence intensity in the absence of quencher, and $F$ is the intensity at each quencher concentration $Q$. The *solid line* was obtained by least-squares analysis of the data.

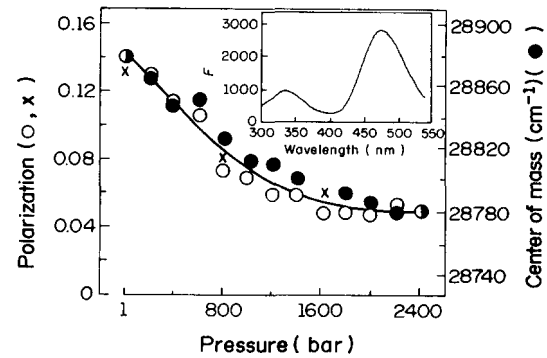


FIG. 3. **Correlation of the effects of pressure on ATPase intrinsic fluorescence emission and polarization of dansyl-ATPase.** ATPase was labeled with 2,5-dansyl in red blood cell ghosts and subsequently purified as described under "Materials and Methods." The *inset* shows fluorescence resonance energy transfer from ATPase tryptophan residues (excitation = 275 nm, peak at 335 nm) to covalently bound dansyl (peak at 415 nm). *F* in the ordinate of the *inset* indicates fluorescence intensity in arbitrary units. Fluorescence polarization measurements (30 μg/ml of ATPase in medium containing 4 mM EDTA) were performed at excitation = 340 nm and emission = 415 nm (○). For comparison, the effect of pressure on the spectral centers of mass of ATPase emission is shown for the same protein concentration (●). Reversibility of the changes in polarization upon release of pressure is also shown (*x*).

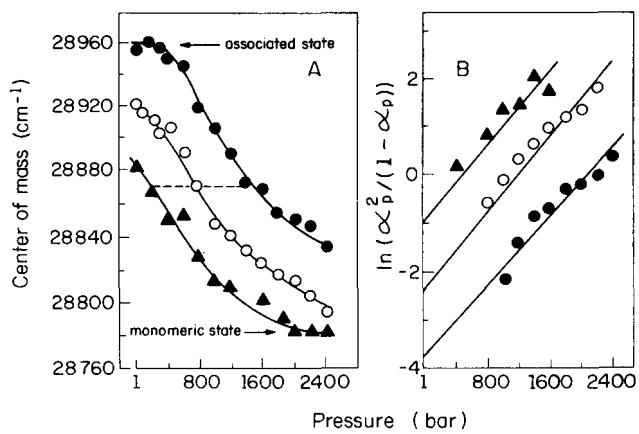


FIG. 4. **Pressure-induced fluorescence red-shift at different ATPase concentrations.** ATPase samples containing 300 (●), 50 (○), and 30 (▲) μg/ml were prepared as described in the legend to Fig. 1. Fluorescence emission spectra were measured from 315 to 420 nm (excitation = 275 nm). Data are presented in terms of spectral centers of mass (*A*) and degrees of dissociation (*B*). Degrees of dissociation were calculated as described in Results (Equation 1), assuming that $\nu_D$ and $\nu_M$ are, respectively, given by the low (28960 $cm^{-1}$) and the high (28780 $cm^{-1}$) pressure plateaus seen in *A* (*arrows*). The *dashed line* in *A* indicates the observed $dp_{1/2}$ (difference in pressure required to promote half-dissociation) for a change in ATPase concentration from 300 to 30 μg/ml. *Straight lines* in *B* were obtained by least-squares analysis of the data.

interface between subunits. In this case, the red shift observed upon dilution and pressure application could be due to rearrangements of the tertiary structure induced by exposure of hydrophobic residues when the protein dissociates.

*Effect of Pressure on the Polarization of Dansyl-ATPase*—Independent proof of the pressure-induced dissociation of the $Ca^{2+}$-ATPase was obtained from measurements of the effect of pressure on the fluorescence polarization of the 2,5-dansyl conjugated enzyme (Fig. 3). The choice of this probe was based on its relatively large lifetime of 30 ns (see "Materials and Methods"), which makes it suitable for reporting the dissociation of oligomeric ATPase. Fig. 3 (*open circles*) shows the polarization values obtained at different pressures. The polarization decreased with increasing pressure up to 1.6 kbar, where a plateau was reached. Since labeling of the ATPase was performed in the native erythrocyte membrane (see "Materials and Methods") we would expect the probe to be attached to lysine residues on the surface of the protein and not at the interface between subunits. Therefore, the decrease in polarization probably reflects a decrease in the size of the rotating particle (Paladini and Weber, 1981).

Fig. 3 (*closed circles*) also shows the red shift of the intrinsic ATPase emission spectra in the same pressure range. As can be seen, the pressure profiles for both polarization of dansyl-ATPase and intrinsic fluorescence emission are exactly parallel. This result confirms that the intrinsic fluorescence emission can be used to follow ATPase dissociation.

*Pressure-induced Fluorescence Red-shift at Different ATPase Concentrations*—The pressure-induced dissociation of oligomeric proteins depends on protein concentration (Weber, 1987), whereas a first-order process such as a conformational change caused by high pressure should be independent of concentration. Fig. 4*A* shows pressure dissociation curves obtained at three different $Ca^{2+}$-ATPase concentrations, 300 μg/ml (*closed circles*), 50 μg/ml (*open circles*), and 30 μg/ml

(*triangles*). Each pressure dissociation curve starts from a different value of center of mass, as expected from the results shown in Fig. 1. At 300 μg/ml (*closed circles*) a plateau at low pressures (1–400 bar) is observed. The final pressure-induced dissociated state, corresponding to the monomer of the ATPase, is achieved only when protein concentration is 30 μg/ml (*triangles*).

The ATPase dissociation data of Fig. 4*A* can be expressed in terms of the degree of dissociation ($\alpha_p$) at each pressure (Weber, 1987), calculated as follows.

$$\alpha_p = (1 + Q\,(\nu_p - \nu_M)/(\nu_D - \nu_p))^{-1} \quad (1)$$

where $\nu_p$ is the center of mass obtained at a given pressure; $\nu_D$ and $\nu_M$ are, respectively, the centers of mass for the associated and monomeric forms obtained from Fig. 4*A*; and $Q$ is the ratio of the fluorescence quantum yields of dissociated and associated forms.

The plot of $\ln[\alpha_p^n/(1 - \alpha_p)]$ as a function of pressure can be described by straight lines at the three ATPase concentrations tested when $n$ is set equal to 2 (Fig. 4*B*). This result indicates that the model of a dimer-monomer equilibrium adequately fits the pressure dissociation data, according to the following equation.

$$\ln[\alpha_p^2/(1 - \alpha_p)] = \ln(K_p/4C) = p\mathrm{d}V^0/RT + \ln(K_0/4C) \quad (2)$$

where $R$ and $T$ have the usual meaning; $K_p$ is the dissociation constant at a given pressure $p$, $K_0$ is the dissociation constant at atmospheric pressure; and $C$ is the molar concentration of the ATPase, expressed as dimer. The intercepts on the $y$ axis of the straight lines provide $K_0$ values of 6–9 × $10^{-8}$ M, in dimer, calculated for the three different protein concentrations. The slopes give the apparent standard volume change for complete association of the monomers ($\mathrm{d}V^0$). The calculated values for $\mathrm{d}V^0$ were 43, 50, and 46 ml/mol for data obtained from 300, 50, and 30 μg/ml, respectively (Fig. 4*B*). That $\mathrm{d}V^0$ values are essentially the same at different protein concentrations further indicates that the three curves shown in Fig. 4*B* correspond to the same dissociation phenomenon. Considering both the fully associated and the monomeric states indicated by the *arrows* in Fig. 4*A* (centers of mass 28960 and 28780 $cm^{-1}$, respectively), it was possible to measure $\mathrm{d}p_{1/2}$. This is the difference between the pressures needed to promote 50% dissociation for a given change in protein concentration from 300 to 30 μg/ml, $\mathrm{d}p_{1/2}$ was found to be 1320 bar (indicated by the *horizontal dashed line* at 28870 $cm^{-1}$ in Fig. 4*A*). The value of $\mathrm{d}p_{1/2}$ is related to $\mathrm{d}V^0$ by the following equation.

$$\mathrm{d}p_{1/2} = \frac{((n - 1)/n)RT \times \ln(C_2/C_1)}{\mathrm{d}V^0/n} \quad (3)$$

where $n$ is the number of subunits of the oligomer and $C_1$ and $C_2$ are the two different ATPase concentrations. Assuming $\mathrm{d}V^0$ = 46 ml/mol and $n$ = 2 (*i.e.* a dimeric structure), a $\mathrm{d}p_{1/2}$ of 1210 bar was predicted. This value is in good agreement with the experimentally determined value of 1320 bar (Fig. 4*A*, *dashed line*), showing that the pattern of dissociation observed for the $Ca^{2+}$-ATPase from red cells corresponds to a dimer/monomer transition. The values calculated for $\mathrm{d}p_{1/2}$ when $n$ was set equal to 3 or 4 were, respectively, 1900 and 2200 bar, which are far from the experimental value found.

Additional support for the monomer-dimer model was obtained from calculation of the Stokes radius of the associated ATPase particle by measuring changes in fluorescence polarization of dansyl-ATPase as a function of the viscosity of the medium. The resulting Perrin plot (Lakowicz, 1983) is shown in Fig. 5. The volume ($V$) of the oligomeric ATPase particle

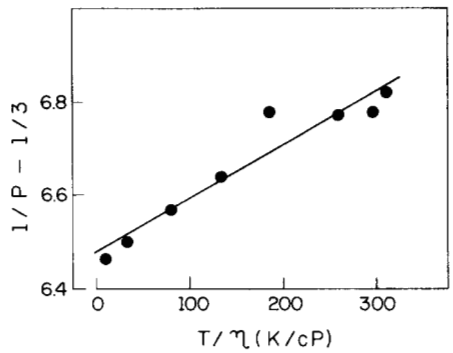


FIG. 5. **Perrin plot of dansyl-ATPase fluorescence polarization.** Dansyl-ATPase samples were diluted to 70 μg/ml in medium containing 4 mM EDTA, 130 mM KCl, 70% sucrose (w/v). The viscosity of the medium was changed by stepwise additions of $Ca^{2+}$-ATPase in the same concentration (70 μg/ml in 4 mM EDTA and 130 mM KCl). This procedure was adopted to avoid changing the state of association of the ATPase, which could otherwise occur upon dilution of the enzyme by additions of sucrose. Dansyl-ATPase fluorescence polarization ($P$) was measured after each addition at excitation and emission wavelengths of 340 and 415 nm, respectively.

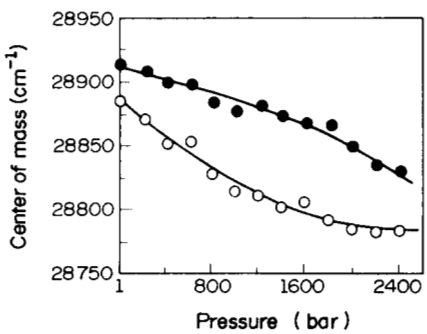


FIG. 6. **Effect of $Ca^{2+}$ on pressure-induced fluorescence red-shift.** ATPase samples were diluted to 30 μg/ml in the absence (4 mM EDTA; ○) or in the presence (●) of 50 μM free $Ca^{2+}$. Emission spectra (excitation = 275 nm) were acquired from 315 to 420 nm.

is calculated by the following equation.

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + (\tau RT/\eta V)) \quad (4)$$

where $R$ and $T$ have the usual meaning; $P_0$ is the limiting polarization; $P$ is the polarization at a given viscosity and $\tau$ is the lifetime of dansyl-ATPase measured as described under "Materials and Methods." The value found for $V$ was 1.4 × $10^6$ ml/mol, corresponding to a Stokes radius of 80 Å. This value is in good agreement with the values of 80 and 75 Å reported in the literature for dimeric sarcoplasmic reticulum Ca-ATPase solubilized in $C_{12}E_8$ (Yamamoto *et al.*, 1984; Verjovski-Almeida *et al.*, 1986).

*Effects of $Ca^{2+}$, Vanadate, and Calmodulin on Pressure-induced Fluorescence Red-shift*—Up to this point all experiments had been performed in the presence of sufficient EDTA to give a free $Ca^{2+}$ concentration below 1 nM. When 50 μM $Ca^{2+}$ was present (Fig. 6, *closed circles*), the effect of pressure on dissociation was significantly attenuated as compared to that observed in the absence of $Ca^{2+}$ (*open circles*). The effects observed in the presence of $Ca^{2+}$ could be due to the increase in $Mg^{2+}$ concentration, which occurs when EDTA is omitted. To exclude this possibility, we assayed the effect of $Mg^{2+}$ alone on pressure dependence curves. When 1 mM $Mg^{2+}$ was added to the medium, the pattern of dissociation of the ATPase remained unmodified as compared to a control sample in the absence of $Mg^{2+}$ (4 mM EDTA; data not shown).

The effect of $Ca^{2+}$ on the dissociation (Fig. 6) suggested

that the equilibrium of association of the ATPase could be affected by the equilibrium between the two major ATPase conformational states $E_1$ and $E_2$. To test this hypothesis, pressure curves were performed in the presence of vanadate (Fig. 7), which binds with high affinity to the ATPase, stabilizing the $E_2$ conformation (Barrabin *et al.*, 1980; Krebs *et al.*, 1987). Comparison of the pressure curves obtained in the absence (Fig. 7, *open circles*) and in the presence (*closed circles*) of vanadate suggests that vanadate further facilitates dissociation in the absence of $Ca^{2+}$.

Pressure dissociation of the ATPase was also carried out in the presence of calmodulin, to investigate whether the activation of the ATPase by calmodulin could be related to changes in intersubunit interactions. Fig. 8 shows the results obtained for dissociation in the presence of $Ca^{2+}$ (*open circles*) or in the presence of $Ca^{2+}$ plus calmodulin (*closed circles*). As can be seen, calmodulin had no additional effect on the dissociation profile as compared to that observed in the presence of $Ca^{2+}$ alone. Pressure dependence profiles presented in Fig. 8 are somewhat different from those shown in Fig. 6 (in the presence of $Ca^{2+}$). This may be explained by the use of excitation at 295 nm in the experiment shown in Fig. 8 to avoid interference from CaM tyrosine fluorescence.

## DISCUSSION

In this study, the association of subunits of detergent-solubilized, purified erythrocyte $Ca^{2+}$-ATPase was investigated through a combination of the use of fluorescence spectroscopy and hydrostatic pressure. Application of pressure up to 2.4 kbar promoted full dissociation of the $Ca^{2+}$-ATPase. The use of these techniques enabled the calculation of the thermodynamic parameters involved in the subunit association of the erythrocyte ATPase, as well as permitted direct measurements of the effects of ligands ($Ca^{2+}$, CaM and vanadate) on the association. In addition, this study extends the observations of pressure-induced dissociation of oligomeric proteins (for reviews, see Heremans, 1982; Weber and Drickamer, 1983; Weber, 1987) to a representative plasma membrane $Ca^{2+}$-ATPase.

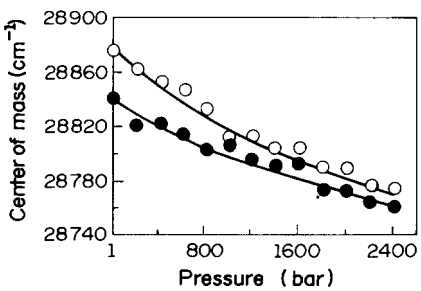


FIG. 7. **Effect of vanadate on pressure-induced fluorescence red-shift.** ATPase samples were diluted to 30 μg/ml (4 mM EDTA) in the absence (○) or in the presence (●) of 50 μM vanadate. Emission spectra (excitation = 275 nm) were acquired from 315 to 420 nm.

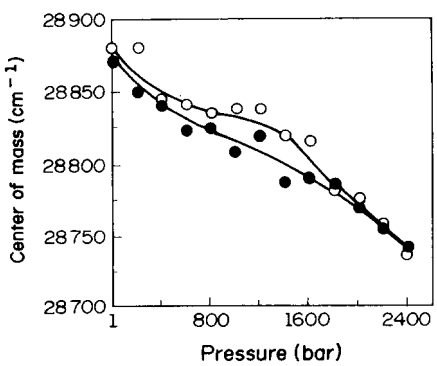


FIG. 8. **Effect of CaM on pressure-induced fluorescence red-shift.** ATPase samples were diluted to 30 μg/ml in medium containing 50 μM $Ca^{2+}$, in the absence (○) or in the presence of (●) 0.5 μM CaM (5:1, in molar ratio to dimeric ATPase). Emission spectra (excitation = 295 nm) were acquired from 320 to 420 nm.

Dissociation constants for dimer-monomer transitions in a number of different proteins reported in the literature range from $10^{-7}$ to $10^{-11}$ M (Paladini and Weber, 1981; Xu and Weber, 1982; Silva *et al.*, 1986; Senear and Ackers, 1988; Ruan and Weber, 1988; Ross *et al.*, 1989; Pin *et al.*, 1990). In this work, the dissociation constant at atmospheric pressure for the erythrocyte $Ca^{2+}$-ATPase was calculated to be in the range of $10^{-8}$ M (Fig. 4). Pressure perturbation of the equilibrium of subunit association was used to dissociate the erythrocyte ATPase at concentrations above the equilibrium constant observed at atmospheric pressure.

Recent studies have made use of enzyme activity and fluorescence energy transfer measurements to investigate the dissociation of erythrocyte $Ca^{2+}$-ATPase by dilution (Kosk-Kosicka and Bzdega, 1988, 1990). These studies indicate that dissociation occurs below a concentration threshold of $3 \times 10^{-8}$ M, while our dilution results indicate that dissociation ocurred at a 10-fold higher protein concentration (Fig. 1). A possible cause of this difference may be the fact that the measurements shown in Fig. 1 were performed in the absence of $Ca^{2+}$, in contrast to those reported by Kosk-Kosicka and Bzdega. As discussed below (see also Kosk-Kosicka *et al.*, 1989), $Ca^{2+}$ is responsible for a significant stabilization of the dimeric form of the ATPase, and, for this reason, it is expected that in the absence of $Ca^{2+}$ (4 mM EDTA, with no $CaCl_2$ added) dissociation occurs at higher protein concentrations than when $Ca^{2+}$ is present. Another point to be considered is that in our dilution experiments the ratio of protein:phospholipid:detergent was maintained constant throughout the range of protein concentrations assayed, whereas Kosk-Kosicka and Bzdega diluted samples in solutions of fixed detergent concentration. This could lead to progressive delipidation of the ATPase as the ratio of protein:detergent decreases. Delipidation could affect the interactions between ATPase subunits in the detergent solution and, consequently, the response to dilution. The studies of Kosk-Kosicka and Bzdega (1988) have shown that the activity of erythrocyte $Ca^{2+}$-ATPase is dependent on detergent concentration in the medium, when ATPase/phospholipid concentrations are held constant.

The dissociation of the $Ca^{2+}$-ATPase by pressure is consistent with a dimer/monomer model. This conclusion is based on the following observations: (i) dissociation data can be adequately fit by a model derived for a dimer/monomer equilibrium (Fig. 4*B*); (ii) in the comparison of data obtained at 300 and 30 μg/ml of ATPase the observed difference of 1320 bar in $dp_{1/2}$ was in very good agreement with the value of 1210 bar predicted assuming the dissociation of a dimer, whereas values of 1900 bar or higher could be calculated for full dissociation of trimers or tetramers; (iii) the Stokes radius of 80 Å obtained from the Perrin plot (Fig. 5) is compatible with a dimeric ATPase particle and is in agreement with the values of 75–80 Å reported for $C_{12}E_8$-solubilized dimeric sarcoplasmic reticulum ATPase (Yamamoto *et al.*, 1984; Verjovski-Almeida *et al.*, 1986).

Analysis of the dissociation curves obtained at different protein concentrations (Fig. 4) enabled us to calculate $dV^0$, the standard volume change upon association. $dV^0$ values of 43–50 ml/mol were calculated for the association of the eryth-

rocyte $Ca^{2+}$-ATPase (Fig. 4*B*). The decrease in volume induced by the application of pressure may result from the occupancy by the solvent molecules of dead spaces present between subunits and/or from better solvation of charged species in the dissociated form (Neumann *et al.*, 1973; Paladini and Weber, 1981). It is interesting to note that our value of 50 ml/mol for the association volume of the erythrocyte ATPase dimer is similar to the value of 65 ml/mol reported for the association of dimeric enolase (Paladini and Weber, 1981) and is significantly smaller than $dV^0$ values reported for the association of the dimers of tryptophan synthase (162 ml/mol; Silva *et al.*, 1986), hexokinase (115–160 ml/mol; Ruan and Weber, 1988), and for sarcoplasmic reticulum $Ca^{2+}$-ATPase in detergent solution (93–167 ml/mol; Verjovski-Almeida *et al.*, 1986). Thus, it may be that, as compared to the latter dimers, intersubunit interactions in erythrocyte $Ca^{2+}$-ATPase are less dependent on electrostatic interactions, the disruption of which is characteristically associated with large standard volume changes (Neumann *et al.*, 1973). Alternatively, erythrocyte ATPase may present a better atomic packing at the intersubunit interface, resulting in decreased dead volumes between subunits.

The erythrocyte $Ca^{2+}$-ATPase alternates between two major conformational states, $E_1$ and $E_2$, during its catalytic cycle (Pedersen and Carafoli, 1987; Krebs *et al.*, 1987; Adamo *et al.*, 1988). Binding of $Ca^{2+}$ shifts the equilibrium to $E_1$ (Adamo *et al.*, 1990), whereas vanadate stabilizes the $E_2$ state (Barrabin *et al.*, 1980; Krebs *et al.*, 1987). In this work, we show that the predominance of one enzyme state over the other affects the monomer/dimer equilibrium. In the presence of $Ca^{2+}$, significantly higher pressures were needed to promote dissociation of dimeric ATPase than in the presence of vanadate (Figs. 5 and 6). This indicates that intersubunit interactions in the ATPase are stronger in the $E_1$ than in the $E_2$ form. These results are in agreement with those of Kosk-Kosicka and co-workers (1989), who reported that $Ca^{2+}$ stabilizes the enzyme against dissociation and inactivation by dilution. These results are also comparable to those reported for sarcoplasmic reticulum ATPase (Verjovski-Almeida *et al.*, 1986), where both inactivation of the enzyme and pressure dissociation are attenuated by an increase in $Ca^{2+}$ concentration.

CaM has been shown to increase the turnover of the erythrocyte $Ca^{2+}$-ATPase (Muallem and Karlish, 1980; Niggli *et al.*, 1981; Adamo *et al.*, 1988). A possible mechanism for this action was proposed to be the acceleration of conversion from $E_2$ to $E_1$ (Muallem and Karlish, 1981; Adamo *et al.*, 1988). However, we show here that CaM (in the presence of $Ca^{2+}$) had no additional effects on the dissociation of the ATPase as compared to that observed with $Ca^{2+}$ alone. It should be noted that, at the free $Ca^{2+}$ concentration used in this study (50 $\mu$M), CaM exerts a significant effect on enzyme activity (3-fold stimulation; Niggli *et al.*, 1981). A recent report (Kosk-Kosicka and Bzdega, 1990) also suggests that CaM is not involved in the equilibrium of association of the ATPase. Our results can be explained by assuming that in fact CaM accelerates the interconversion between $E_1$ and $E_2$ in both directions, instead of favoring the equilibrium in only one direction as originally proposed by Adamo and co-workers (1988). It may also be that the equilibrium between conformers could be maximally shifted toward $E_1$ by the addition of $Ca^{2+}$, and for this reason there is no additional effect of calmodulin. There is evidence that depending on incubation conditions micromolar $Ca^{2+}$ concentrations accelerate the initial rate of phosphorylation by ATP of the native $Ca^{2+}$-ATPase from red cells, an effect attributed to a change in the distribution between conformers in favor of $E_1$ (Adamo *et al.*, 1990). In another P-type ATPase, the $Ca^{2+}$-ATPase from sarcoplasmic reticulum, transient state kinetic studies indicate that micromolar $Ca^{2+}$ concentrations also stabilize $E_1$ (Vieyra *et al.*, 1979; Scofano *et al.*, 1979).

In conclusion, this work indicates that the detergent-solubilized $Ca^{2+}$-ATPase from erythrocyte can be dissociated from a dimeric to a monomeric state by application of hydrostatic pressure. Dissociation by pressure is favored in the $E_2$ ATPase state and is more difficult when the equilibrium is shifted to the $E_1$ state, suggesting that intersubunit interactions vary along the reaction cycle of the ATPase.

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