described here may have some physiological relevance, i.e., the possible substrate-induced loss in catalytic efficiency of enzymes in cells.

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REFERENCES

Arnone, A., Rogers, P. H., Hyde, C. C., Briley, P. O., Metzler, C. M., & Metzler, D. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 138, Wiley, New York.

Churchich, J. E., & Farrelly, J. G. (1969) *J. Biol. Chem. 244*, 72–76.

Fisher, J., Charnas, R. L., & Knowles, J. R. (1978) *Biochemistry 17*, 2180–2184.

Frere, J. M., Dormans, C., Duychaerts, C., & DeGraeve, J. (1982) *Biochem. J. 207*, 437–444.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. (1981) in *The Molecular Basis of Antibiotic Action*, Wiley, New York.

Jones, W. M., Soper, T. S., Ueno, H., & Manning, J. M. (1985) *Methods Enzymol. 113*, 108.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Likos, J. J., Ueno, H., Feedhaus, R. W., & Metzler, D. E. (1982) *Biochemistry 21*, 4377.

Manning, J. M., Merrifield, N. E., Jones, W. M., & Gotschlich, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 417–421.

Martinez-Carrion, M., & Jenkins, W. T. (1965) *J. Biol. Chem. 240*, 3538.

Martinez del Pozo, A., Merola, M., Ueno, H., Manning, J. M., Tanizawa, K., Nishimura, K., Asano, S., Tanaka, H., Soda, K., Ringe, D., & Petsko, G. A. (1989a) *Biochemistry 28*, 510.

Martinez del Pozo, A., Pospischil, M. A., Ueno, H., Manning, J. M., Tanizawa, K., Nishimura, K., Soda, K., Ringe, D., Stoddard, B., & Petsko, G. A. (1989b) *Biochemistry 28*, 8798.

Merola, M., Martinez del Pozo, A., Ueno, H., Recsei, P., Di Donato, A., Manning, J. M., Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., Soda, K., Ringe, D., & Petsko, G. A. (1989) *Biochemistry 28*, 505.

Novogrodsky, A., & Meister, A. (1964) *J. Biol. Chem. 239*, 879–888.

Soper, T. S., & Manning, J. M. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 266, Wiley, New York.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.

Sukhareva, B. S., & Braunstein, A. E. (1971) *Mol. Biol. (Moscow) 5*, 302.

Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, K., Kagamiyama, N., Tanaka, H., & Soda, K. (1989a) *J. Biol. Chem. 264*, 2450.

Tanizawa, K., Masu, Y., Asano, S., Tanaka, H., & Soda, K. (1989b) *J. Biol. Chem. 264*, 2445.

Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089.

Waley, S. G. (1991) *Biochem. J. 279*, 87–94.

Labeling the (Ca^{2+}–Mg^{2+})-ATPase of Sarcoplasmic Reticulum with 4-(Bromomethyl)-6,7-dimethoxycoumarin: Detection of Conformational Changes†

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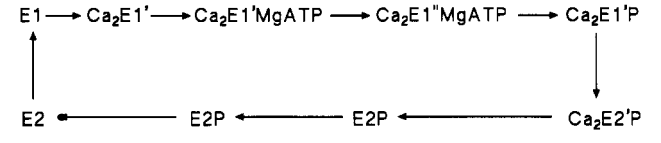
ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum was labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin. It was shown that a single cysteine residue (Cys-344) was labeled on the ATPase, with a 25% reduction in steady-state ATPase activity and no reduction in the steady-state rate of hydrolysis of *p*-nitrophenyl phosphate. The fluorescence intensity of the labeled ATPase was sensitive to pH, consistent with an effect of protonation of a residue of p*K* 6.8. Fluorescence changes were observed on binding Mg^{2+}, consistent with binding to a single site of K_d 4 mM. Comparable changes in fluorescence intensity were observed on binding ADP in the presence of Ca^{2+}. Binding of AMP-PCP produced larger fluorescence changes, comparable to those observed on phosphorylation with ATP or acetyl phosphate. Phosphorylation with P_i also resulted in fluorescence changes; the effect of pH on the fluorescence changes was greater than that on the level of phosphorylation measured directly using [^{32}P]P_i. It is suggested that different conformational states of the phosphorylated ATPase are obtained at steady state in the presence of Ca^{2+} and ATP and at equilibrium in the presence of P_i and absence of Ca^{2+}.

One approach to the study of enzyme function is to attach a fluorescent probe to a particular residue in the enzyme and to use the spectral properties of the probe to report on changes in its environment during the reaction cycle of the enzyme. This approach has been used to study the function of the (Ca^{2+}–Mg^{2+})-ATPase from skeletal muscle sarcoplasmic reticulum (SR),[1] the major problem being that of ensuring labeling at a single, specific residue on the ATPase. Studies have

Scheme I



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been interpreted in terms of the reaction scheme shown in simplified form in Scheme I. It is proposed that the ATPase can exist in one of two main conformational states E1 or E2 and that in the E1 state the ATPase has two high-affinity binding sites for Ca^{2+}, exposed on the cytoplasmic side of the SR membrane, and a binding site for MgATP (de Meis & Vianna, 1979). Following phosphorylation of the ATPase at Asp-351, the ATPase transforms into the E2P state from which Ca^{2+} is lost into the lumen of the SR. The ATPase can then recycle to E1.

It has been shown that the ATPase can be labeled with fluorescein isothiocyanate specifically at Lys-515 in the ATP binding site (Pick & Karlish, 1980; Mitchinson et al., 1982). The fluorescence of the labeled ATPase has been shown to be sensitive to the E2–E1 transition (Pick & Karlish, 1980; Pick, 1981a; Froud & Lee, 1986a) and to phosphorylation of the ATPase by P_i (Pick, 1981b). A variety of sulfhydryl reagents have also been used to label the ATPase. The (Ca^{2+}–Mg^{2+})-ATPase contains 24 cysteinyl residues (MacLennan et al., 1985), of which about 14 are on the surface of the protein, and which show a wide range of reactivities to sulfhydryl reagents (Murphy, 1976; Yamada & Ikemoto, 1978; Yamashita & Kawakita, 1987). Iodoacetamide and 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonate (IAEDANS) have been reported to label a single cysteinyl residue, Cys-674 (Suzuki et al., 1987; Yamashita & Kawakita, 1987), although Bishop et al. (1988) reported labeling of both Cys-670 and Cys-674. *N*-Ethylmaleimide has been found to label both Cys-344 and Cys-364 (Kawakita & Yamashita, 1987; Saito-Nakatsuka et al., 1987). Wakabayashi et al. (1990a) have shown that labeling the ATPase with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) in the presence of Ca^{2+} and a nonhydrolyzable analogue of ATP followed by treatment with dithiothreitol to remove some of the NBD label gave a preparation containing essentially a single modified residue, Cys-344. The fluorescence of the labeled ATPase was found to be sensitive to the E2–E1 transition and to binding of Ca^{2+} and ATP (Wakabayashi et al., 1990b). Changes in fluorescence on phosphorylation by ATP or P_i were, however, low and the ATPase activity was only about 10% of that of the unmodified ATPase (Wakabayashi et al., 1990a,b).

It has been suggested that the pattern of labeling observed by sulfhydryl reagents is determined in large part by charge (Bishop et al., 1988). Relatively polar reagents such as iodoacetamide or IAEDANS label the ATPase in a region close to cationic Arg residues, whereas more apolar reagents such as *N*-ethylmaleimide label the ATPase at Cys residues in a relatively apolar region of the ATPase, in the phosphorylation domain (Bishop et al., 1988). The apolar coumarin derivative 4-(bromomethyl)-7-methoxycoumarin (Br-DMC) was introduced for labeling the carboxyl groups of fatty acids in organic solvents (Dungee, 1977) but has also been shown to react with sulfhydryl groups (Hiratsuka, 1987). Here we show that Br-DMC specifically labels the (Ca^{2+}–Mg^{2+})-ATPase at Cys-344, with a fluorescence sensitivity very different from that reported for the NBD-labeled ATPase.

Materials and Methods

4-(Bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) was obtained from Molecular Probes. Sarcoplasmic reticulum from rabbit skeletal muscle and the purified (Ca^{2+}–Mg^{2+})-ATPase were prepared as described in East and Lee (1982). ATPase activity was determined at 25 °C by using a coupled enzyme assay in a medium, unless otherwise specified, containing 40 mM Hepes/KOH (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 0.41 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 mL, with $CaCl_2$ and EGTA added to give a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration of ca. 10 μM). The reaction was initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents.

The rate of hydrolysis of *p*-nitrophenyl phosphate (pNPP) was determined essentially as described in Rossi et al. (1979). Assays were performed in a solution of 40 mM Hepes/KOH, 100 mM KCl, 5 mM $MgSO_4$, pH 7.4, optimal Ca^{2+}, and 1–20 mM pNPP. Liberation of *p*-nitrophenol was monitored at 410 nm using a molar extinction coefficient of 6000 M^{-1} cm^{-1}.

Ca^{2+} uptake was measured photometrically by using the dye murexide (McWhirter et al., 1987). Spectra were run on a SLM-Aminco DW2000 spectrophotometer with a wavelength pair of 507 nm–542 nm. A saturated solution of murexide was prepared by addition of 2.4 mg of murexide to 1 mL of water, leaving the resulting suspension to stand on ice for 30 min, followed by filtration to give a clear solution. For Ca^{2+} assay, 160 μL of this stock solution was added to 3 mL of buffer containing 0.3 mg/mL SR in the assay cuvette, at 25 °C. To calibrate the optical signal, samples of a concentrated stock solution of $CaCl_2$ were added, to a final concentration of 50 μM. Ca^{2+} uptake was initiated by addition of ATP.

Phosphorylation of the (Ca^{2+}–Mg^{2+})-ATPase by [^{32}P]P_i was performed largely as described by Martin and Tanford (1981). SR (0.1 mg) was incubated in 40 mM Tris/maleate, pH 6.4 or 7.0, 20 mM Mg^{2+}, 1 mM EGTA, and the reaction was started by addition of $H_2{}^{32}PO_4$ (Amersham, 5 Ci mol^{-1}) to give a range of final concentrations of 1–10 mM P_i in a final volume of 300 μL. The mixture was quenched after 20 s with 1.0 mL of 15% trichloroacetic acid and 0.2 M phosphoric acid and then filtered through a Whatman GF/C filter and washed with 3 × 15 mL of the quenching solution. The filter was then dried, and the extent of phosphorylation was determined by liquid scintillation counting. Nonspecific binding of [^{32}P]P_i on the glass fiber filters was assessed by adding the quench solution to the assay mix before adding the radiolabel and then washing and counting the sample as above. Nonspecific binding was typically 10% of total counts. Concentrations of free P_i and Mg^{2+} were calculated using binding constants of 50 and 5 × 10^6 for Mg^{2+} + HPO_4^{2-} and H^+ + HPO_4^{2-}, respectively (Smith & Martell, 1976).

Labeling with Br-DMC. SR or purified ATPase was suspended to 8 mg/mL in buffer (50 mM Tris/HCl, pH 7.0, 200 mM sucrose) at room temperature and incubated with 1 mM Br-DMC in the dark for 1 h; Br-DMC was added from a 20 mM stock solution in dimethylformamide. Unbound Br-DMC was separated from the labeled ATPase by centrifugation through Sephadex G-50 columns preequilibrated with the above buffer (Munkonge et al., 1989). Concentrations of ATPase and SR were estimated from the absorbance at 280 nm in 1% SDS, 5 mM KOH, 15% methanol, using the extinction coefficients given by Hardwicke and Green (1974), and the amount of DMC covalently bound to the ATPase was estimated from the absorbance at 350 nm, using an extinction coefficient of 12900 M^{-1} cm^{-1} (Hiratsuka, 1987).

Proteolysis of the ATPase and Separation of Peptides. Labeled ATPase (1.6 mg/mL) was incubated with 16 μg of thermolysin (Sigma, protease type X) in 0.125 M Tris/HCl,

[1] Abbreviations: AMP-PCP, adenosine 5′-[β,γ-methylene]triphosphate; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; pNPP, *p*-nitrophenyl phosphate; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid.

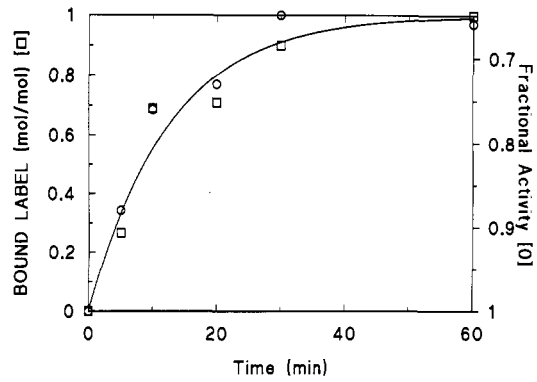

FIGURE 1: Modification of the ATPase by Br-DMC. The (Ca^{2+}–Mg^{2+})-ATPase (80 μM) was incubated with Br-DMC (1 mM) at pH 7.0, 25 °C, for the given times, and unreacted Br-DMC was separated from labeled protein on Sephadex G-50. The ratio of bound label to ATPase (□) was estimated by absorbance. ATPase activities were measured at pH 7.2, 100 mM KCl, 25 °C, 2.1 mM ATP and maximally stimulating concentrations of Ca^{2+} is described in Materials and Methods, and are expressed as a fraction of that measured for the unmodified ATPase (○). The line shows a fit to a single exponential with a first-order rate constant of 0.084 min^{-1}.

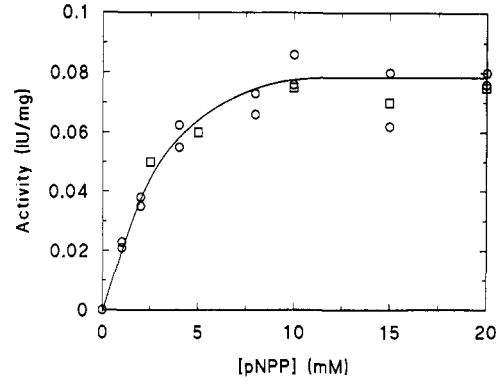

FIGURE 2: Effect of modification of the ATPase by Br-DMC on the rate of hydrolysis of *p*-nitrophenyl phosphate. The data show *p*-nitrophenyl phosphatase activity for unlabeled ATPase (○) and for the DMC–ATPase (□), at 25 °C, pH 7.4, and the given concentrations of pNPP.

pH 6.8, in a total volume of 250 μL. The digestion was terminated after 2 h by centrifugation at 14000*g* for 2 min. The supernatant was analyzed for peptide and coumarin by absorbance measurements at 235 and 350 nm, respectively. As estimated from the absorbance, 80% of the coumarin label was present in the supernatant.

Separation of peptides was performed using an Applied Biosystems gradient HPLC system, Model 1783A, equipped with a variable wavelength absorbance detector and an Applied Biosystems 980 fluorescence detector. Peptides were purified by reverse-phase chromatography using 25 × 0.4 cm octadecyl columns purchased from Jones Chromatography, using a gradient made from 0.1% TFA in water and 0.1% TFA in methanol or from 5 mM potassium phosphate and 20 mM K_2SO_4 made to pH 6.9 with TFA and 0.1% TFA in methanol.

Peptide Sequencing. Amino acid sequences of DMC–peptides were determined using a pulse-liquid sequencer (Applied Biosystems, Model 477A) equipped with an on-line PTH analyzer.

Fluorescence Measurements. Fluorescence measurements were performed at 25 °C using an SLM-Aminco 8000C fluorometer with excitation at 350 nm and emission at 425 nm. Samples contained 1 μM ATPase in 40 mM Tris/maleate, 1 mM EGTA, unless otherwise stated. Stock solutions of P_i and EGTA were prepared by neutralizing orthophosphoric acid and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid with Tris. Ammonium vanadate was dissolved in KOH (100 mM) to give a 100 mM stock solution and was added to the fluorescence samples to give a final concentration of 100 μM. All measurements were corrected for dilution.

RESULTS

Modification of (Ca^{2+}–Mg^{2+})-ATPase by Br-DMC. When the purified (Ca^{2+}–Mg^{2+})-ATPase was incubated with 1 mM Br-DMC at 25 °C for up to 60 min, coumarin label was incorporated into the ATPase up to a 1:1 molar ratio of label to ATPase (Figure 1). Steady-state ATPase activity decreased in parallel with the incorporation of coumarin label, to give, at a 1:1 molar ratio of label to ATPase, an activity 75% of that of the unmodified ATPase (Figure 1). Both the extent of incorporation of label and the decrease in ATPase activity varied monoexponentially with time, described by a rate constant of 0.084 min^{-1}. The pattern of labeling was unaffected by inclusion of 2.5 mM ATP during the incubation with Br-DMC. The dependence of steady-state ATPase activity on the concentrations of Ca^{2+} and ATP was unaffected by modification with Br-DMC (data not shown). In contrast to the inhibition of ATP hydrolysis, the rate of hydrolysis of *p*-nitrophenyl phosphate by the ATPase was unaffected by modification (Figure 2).

The effect of modification of the ATPase by Br-DMC on Ca^{2+} uptake by SR vesicles was studied spectrophotometrically, using murexide to monitor the concentration of Ca^{2+} in the bulk medium. Addition of ATP to SR vesicles in the presence of Ca^{2+} leads to a rapid accumulation of Ca^{2+} followed by spontaneous release of some of the accumulated Ca^{2+} (McWhirter et al., 1987). For the preparations of SR used here, the maximal observed level of Ca^{2+} accumulation was 210 nmol of Ca^{2+}/mg of protein, as reported previously (McWhirter et al., 1987). Modification of SR with Br-DMC up to a 1:1 molar ratio of incorporated label:ATPase had no significant effect either on the maximal level of Ca^{2+} uptake or on the spontaneous release of Ca^{2+} observed following uptake (data not shown).

Identification of Residue Modified by Br-DMC. The purified (Ca^{2+}–Mg^{2+})-ATPase was incubated with 1 mM Br-DMC for 60 min, and excess Br-DMC was removed by two passages through Sephadex G-50. The ATPase was then partially digested with thermolysin, and soluble peptides were separated from membranous remnants by centrifugation. As estimated from measurements of absorbance at 350 nm, about 80% of the bound coumarin was recovered in the supernatant. The peptides in the supernatant were analyzed by reverse-phase HPLC with a methanol gradient in 0.1% trifluoroacetic acid (Figure 3). Elution of peptides (and other material) was followed by far-UV absorbance at 235 nm, and elution of coumarin label was followed by fluorescence, using excitation and emission wavelengths of 350 and 425 nm, respectively. As shown in Figure 3, coumarin fluorescence was detected in five major peaks. Unreacted Br-DMC was shown in separate experiments to give peaks eluting at 9, 11.3, and 17 min, corresponding to peaks 1, 2, and 4 in Figure 3. Peaks 3 and 5 were collected and, as estimated from absorbance measurements at 350 nm, contained 19% and 45%, respectively, of the total coumarin label applied to the HPLC column. Peaks 3 and 5 constitute ca. 70% of the total fluorescence detected in the HPLC trace shown in Figure 3, with peaks 1, 2, and 3 constituting ca. 30% of the total fluorescence, with negligible amounts of fluorescence present in others peaks (<10% in total). It is likely that the free coumarin was formed by nucleophilic attack on the DMC–cysteine adduct as re-

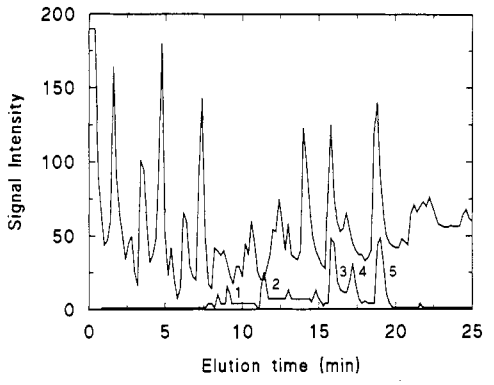

FIGURE 3: Analysis of peptide fragments by reversed-phase HPLC. Soluble peptides obtained by treatment of DMC–ATPase with thermolysin were fractionated on a C_{18} column at a flow rate of 1 mL/min. The peptides were eluted with a linear gradient of methanol in 0.1% trifluoroacetic acid, from 35% to 60% methanol. The top trace shows absorbance measured at 235 nm, and the lower trace shows fluorescence, measured with excitation and emission wavelengths of 350 and 425 nm, respectively.

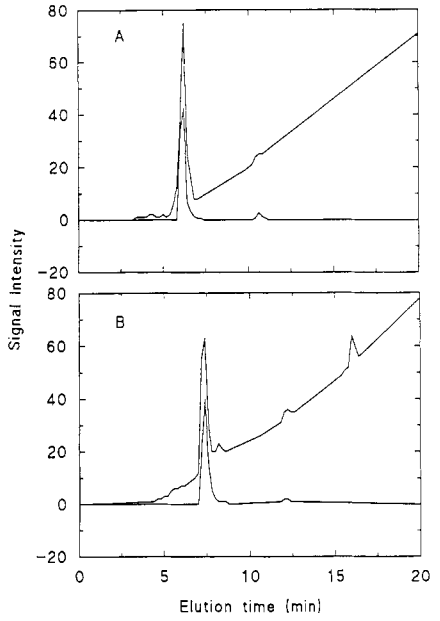

FIGURE 4: Purification of peptide fragments by reversed-phase HPLC. Peaks 3 (A) and 5 (B) obtained as shown in Figure 3 were further fractionated on a C_{18} column at a flow rate of 1 mL/min, with a linear gradient of methanol in 5 mM potassium phosphate and 20 mM K_2SO_4 made up to pH 6.9 with TFA, from 40% to 60% methanol. The top trace shows absorbance measured at 235 nm, and the lower trace shows fluorescence, measured with excitation and emission wavelengths of 350 and 425 nm, respectively.

ported for the NBD–cysteine adduct (Wakabayashi et al., 1990a).

The peptides in peaks 3 and 5 were further purified by reverse-phase HPLC with a methanol gradient in 5 mM potassium phosphate (pH 6.9) and 20 mM Na_2SO_4. As shown in Figure 4, both fractions 3 and 5 contained essentially single peptides. Samples from the column were collected and sequenced, and the results are shown in Table I.

Fluorescence Properties of DMC–ATPase. The (Ca^{2+}–Mg^{2+})-ATPase labeled with Br-DMC exhibits fluorescence emission centered at 440 nm with an excitation maximum of 320 nm, attributable to the coumarin fluorophore. Identical fluorescence responses are obtained following labeling of either the purified (Ca^{2+}–Mg^{2+})-ATPase or SR vesicles with Br-DMC, and labeling both with an excess of Br-DMC results in a labeling ratio of 1 DMC per ATPase molecule. We

Table I: Sequences of Purified DMC–Peptides

peptide peak[a]	amino acid sequence and assignment[b]
3	$Leu_{342}Gly_{343}XThr_{345}Ser_{346}$
5	$Leu_{342}Gly_{343}X$

[a] Peaks 3 and 5 from the HPLC run shown in Figure 3 were further purified as shown in Figure 4 and sequenced. [b] X represents the position of a PTH-amino acid derivative which elutes from the reverse-phase column in the same position as PTH-tryptophan. Examination of the published sequence of the ATPase (Brandl et al., 1986) shows this residue to be derived from Cys-344.

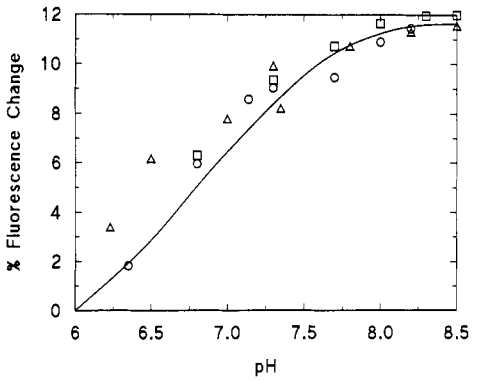

FIGURE 5: Effect of pH on the fluorescence intensity of DMC–ATPase. DMC–ATPase was incubated in 40 mM Tris/maleate, pH 6.0, 1 mM EGTA, and the pH was changed to the given values by addition of solid Tris (○). The experiment was repeated in the presence of 20 mM Mg^{2+} (□) or 100 μM Ca^{2+} and no EGTA (△). The solid line shows the percentage fluorescence change calculated for a pK of 6.8, with a fluorescence change from protonated to unprotonated species of 14%.

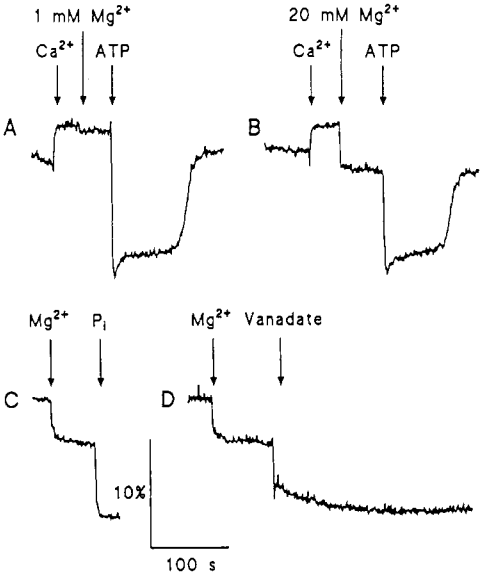

FIGURE 6: Changes in the steady-state fluorescence intensity of DMC–SR. DMC–SR was incubated in 40 mM Tris/maleate, pH 6.0, 1 mM EGTA. In (A) and (B), following addition of Ca^{2+} to give a free Ca^{2+} concentration of 100 μM, either 1 mM (A) or 20 mM (B) Mg^{2+} was added, followed by 10 μM ATP. (C): Addition of 20 mM Mg^{2+} followed by 20 mM P_i. (D): Addition of 20 mM Mg^{2+} followed by 100 μM vanadate.

conclude that only the (Ca^{2+}–Mg^{2+})-ATPase is labeled in SR vesicles.

Although the fluorescence of free Br-DMC is unaffected by pH, the fluorescence intensity of DMC–ATPase is pH sensitive, as shown in Figure 5. In the experiment shown, DMC–ATPase was incubated in pH 6.0 buffer, and the pH was changed by addition of solid Tris. On changing pH from 6 to 8.5, fluorescence intensity increased by 12%, and, as

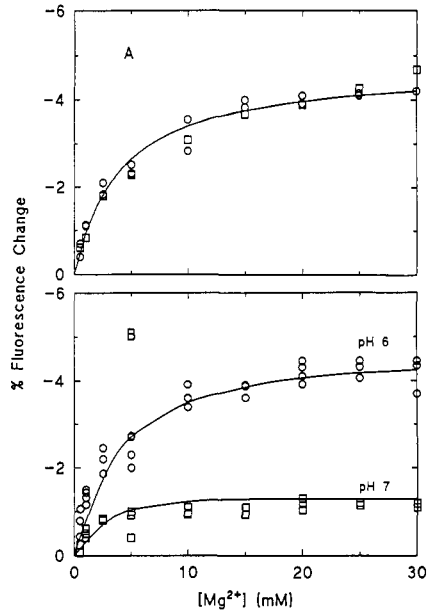

FIGURE 7: Effect of Mg^{2+} on the steady-state fluorescence intensity of DMC–SR. DMC–SR was incubated in 40 mM Tris/maleate at pH 6.0 (○) or 7.0 (□) containing either 100 μM free Ca^{2+} (A) or 1 mM EGTA (B). The change in fluorescence intensity (%) is shown as a function of the concentration of Mg^{2+}, after correction for the volume change caused by each addition. The solid lines are simulations with a K_d of 4 mM for Mg^{2+} binding, as described in the text.

shown, the data were fitted to a single ionization, with pK 6.8 and a fluorescence change from protonated to unprotonated species of 14%.

Figure 6 shows the fluorescence response of SR vesicles labeled with Br-DMC (DMC–SR) to the addition of Ca^{2+}, Mg^{2+}, and ATP at pH 6.0. As shown, addition of 100 μM Ca^{2+} results in a 3% increase in fluorescence intensity at pH 6.0. At pH 7.0 and 8.0, addition of Ca^{2+} results in a 1% increase and a 1% decrease in fluorescence intensity, respectively (data not shown). Addition of Mg^{2+} following the addition of Ca^{2+} results in a decrease in fluorescence intensity of up to 4% (Figures 6 and 7A). The dependence of the fluorescence change on the concentration of Mg^{2+} (Figure 7) fits to binding of Mg^{2+} to a single binding site, with a K_d of 4.0 ± 0.5 mM at pH 6 and 4.6 ± 0.6 mM at pH 7.0. The effect of Mg^{2+} on fluorescence intensity in the absence of Ca^{2+} is pH dependent, with a smaller change in fluorescence intensity at pH 7.0 than at pH 6.0 (Figure 7B). The apparent K_d for binding of Mg^{2+} also changes, the data fitting to values of 4.0 ± 0.54 mM and 1.8 ± 0.42 mM at pH 6.0 and 7.0, respectively.

Addition of ATP to DMC–ATPase in the presence of Ca^{2+} and Mg^{2+} results in a further decrease in fluorescence intensity, which reverses after ca. 100 s, due to hydrolysis of the added ATP by the ATPase (Figure 6). As shown, the total change seen on addition of Mg^{2+} and ATP is constant; the decrease in fluorescence intensity following addition of Mg^{2+} and ATP to DMC–SR in the presence of Ca^{2+} is ca. 13%, for either 1 mM or 20 mM Mg^{2+} (Figure 6).

Following hydrolysis of the added ATP, a final equilibrium level of fluorescence is reached which is lower than the initial level of fluorescence. This can be attributed to binding of the ADP generated by hydrolysis of ATP to the ATPase. As shown in Figure 8, the decrease in fluorescence intensity following addition of ATP to DMC–SR in the presence of Ca^{2+} and Mg^{2+} is constant at added ATP concentrations between 3.3 and 50 μM, but the time taken to hydrolyze the added ATP increases, and the final equilibrium level of

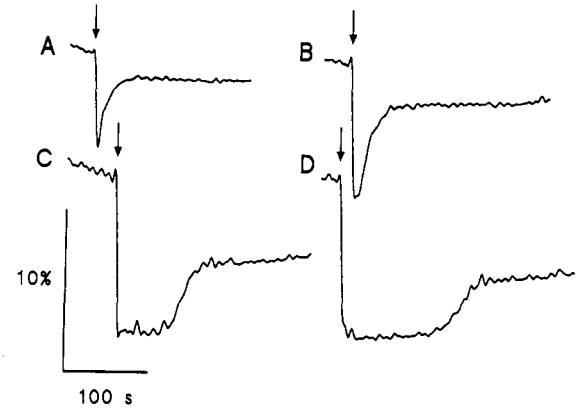

FIGURE 8: Effect of ATP on the fluorescence intensity of DMC–SR. DMC–SR was incubated in 40 mM Hepes/KOH, pH 7.0, 100 mM KCl, 50 μM Ca^{2+}, 5 mM Mg^{2+}, and ATP was added to initial micromolar concentrations of (A) 1, (B) 3.3, (C) 25, and (D) 50.

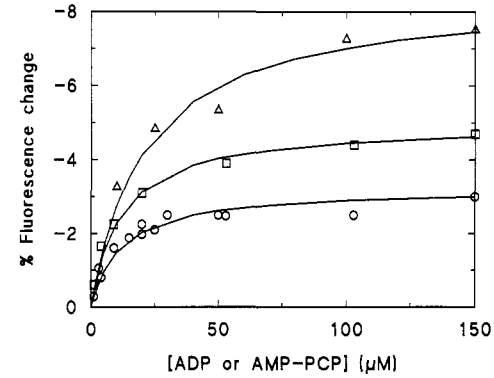

FIGURE 9: Effect of ADP and AMP-PCP on the fluorescence intensity of DMC–SR. DMC–SR was incubated in 40 mM Hepes/KOH, pH 7.0, 100 mM KCl, 50 μM Ca^{2+}, in the presence (△, ○) or absence (□) of 5 mM Mg^{2+} and the decrease in fluorescence intensity was measured on addition of ADP (○, □) or AMP-PCP (△).

fluorescence also decreases. The smaller decrease in fluorescence intensity resulting from addition of 1 μM ATP can be attributed to significant hydrolysis of the ATP before all the ATPase is phosphorylated. Figure 9 shows the decrease in fluorescence intensity for DMC–SR observed on addition of ADP in the presence of Ca^{2+}. The data fits to a single binding constant of K_d 9.4 ± 1.5 μM in the presence or absence of Mg^{2+}, with a maximal decrease in fluorescence intensity of 3% and 4.7% in the presence and absence of 5 mM Mg^{2+}, respectively (Figure 9); as described above for ATP, the total change seen on addition of Mg^{2+} and ADP is constant between 1 and 20 mM Mg^{2+} (data not shown). Equilibrium fluorescence levels were obtained immediately following addition of ADP demonstrating the lack of any ATP contamination in the ADP sample (or of generation of ATP by any contaminating adenylate kinase). A greater decrease in fluorescence intensity is observed on addition of the nonhydrolyzable ATP analogue adenosine 5′-[β,γ-methylene]triphosphate (AMP-PCP) to DMC–SR in the presence of Ca^{2+}, the data fitting to a single site with K_d 21.1 ± 4.2 μM (Figure 9), again with the total change on addition of Mg^{2+} and AMP-PCP being constant between 1 and 20 mM Mg^{2+} (data not shown). At pH 6, identical changes are seen on addition of ADP or AMP-PCP in the presence or absence of Ca^{2+}, but at pH 7, effects of ADP or AMP-PCP are very much smaller in the absence of Ca^{2+} than in its presence (data not shown), paralleling the effects of pH on the response to Mg^{2+} in the absence of Ca^{2+} (Figure 7B). Thus, Figure 10A shows an experiment in which EGTA is added to DMC–SR at pH 7.0

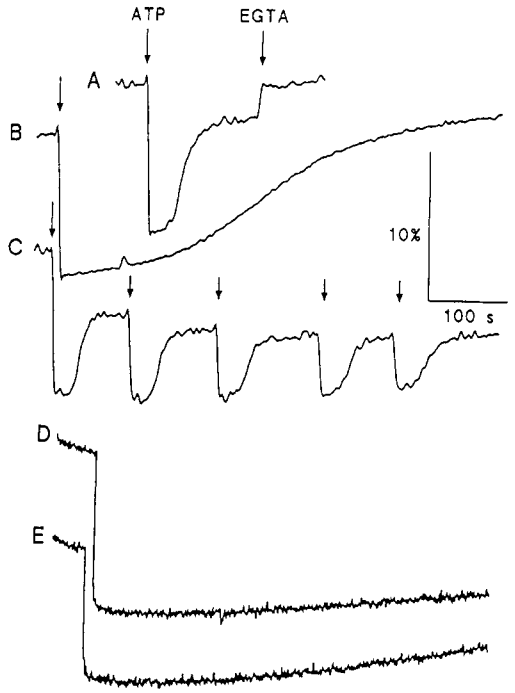

FIGURE 10: Effect of phosphorylation on the fluorescence intensity of DMC–SR. In (A), (B), and (C), DMC–SR was incubated in 40 mM Hepes/KOH, pH 7.0, 100 mM KCl, 5 mM Mg^{2+}, 50 μM Ca^{2+}. In (A), following the addition of 10 μM ATP, 1 mM EGTA was added to complex free Ca^{2+}. In (B), a regenerating system of pyruvate kinase (7 IU) and 0.1 mM phosphoenolpyruvate was present. In (C), 10 μM aliquots of ATP were added as shown by the arrows. In (D) and (E), DMC–ATPase was incubated in 40 mM Tris/maleate, 50 μM Ca^{2+}, 5 mM Mg^{2+} at either pH 6.0 (D) or pH 7.0 (E). The response is shown to the addition of 0.5 mM acetyl phosphate.

following the complete hydrolysis of added ATP, and, as shown, this results in complete reversal of the fluorescence change.

As shown in Figure 6, addition of P_i or vanadate to DMC–ATPase at pH 6 in the presence of Mg^{2+} and absence of Ca^{2+} results in a decrease in fluorescence intensity comparable to that seen with ATP. Addition of acetyl phosphate to the ATPase in the presence of Ca^{2+} also results in a decrease in fluorescence intensity, comparable to that seen with ATP (Figure 10). The decrease in fluorescence intensity seen immediately following addition of ATP in the presence of Ca^{2+} could either follow directly from binding of ATP to DMC–ATPase (if the effect of ATP was comparable to that of AMP-PCP) or could follow from phosphorylation of the ATPase; the steady-state level of fluorescence must correspond to the formation of phosphorylated ATPase, since the ATPase is predominantly present in a phosphorylated form under these conditions [see Michelangeli et al. (1991)]. Since binding of ADP to the ATPase in the presence of Ca^{2+} results in a decrease in fluorescence intensity (Figure 9), it is important to establish whether the steady-state level of fluorescence observed in the presence of ATP is affected by binding of ADP generated by hydrolysis of the added ATP. Figure 10C shows the result of an experiment in which sequential additions of ATP were made to DMC–SR. The maximal decrease in fluorescence intensity remained constant in this experiment, although the equilibrium level of fluorescence reached after hydrolysis of each added aliquot of ATP decreased due to the generation of ADP. Figure 10B shows an experiment in which ATP was added in the presence of an ATP-regenerating system, to prevent the formation of ADP. The maximal decrease in fluorescence intensity observed in this experiment was identical to that in the absence of a regenerating system.

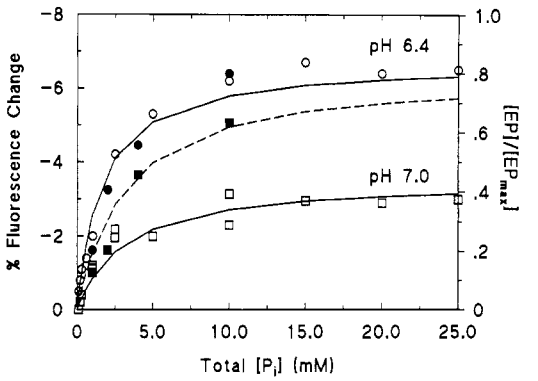

FIGURE 11: Effect of phosphorylation by P_i on the fluorescence intensity of DMC–SR. DMC–SR was incubated in 40 mM Tris/maleate, pH 6.4 (○) or 7.0 (□), 20 mM Mg^{2+}, 1 mM EGTA, and the fluorescence response to the addition of P_i was recorded. Phosphorylation by [^{32}P]P_i was also measured at pH 6.4 (●) and pH 7.0 (■) and is expressed (right-hand axis) as [EP]/[EP_{max}], where [EP_{max}] = 3.4 nmol of EP/mg of protein. The solid lines show simulations of the change in fluorescence intensity, calculated as described in the text, and the broken line shows a simulation of EP formation at pH 7.0, calculated as described in the text.

We therefore conclude that binding of ADP does not contribute to the decrease in fluorescence intensity observed following addition of ATP in the presence of Ca^{2+}.

The fluorescence intensity of the phosphorylated DMC–ATPase formed from ATP is unaffected by binding of Ca^{2+} at luminal sites (to give Ca_2E2P in Scheme I) since the fluorescence intensity in DMC–SR is unaffected by addition of the calcium ionophore BrA23187 to lower the luminal concentration of Ca^{2+}, and since fluorescence changes are the same for the purified ATPase and for sealed SR vesicles (data not shown).

Addition of P_i to DMC–SR in the absence of Mg^{2+} has no effect on fluorescence intensity but in the presence of Mg^{2+} at pH 6.4, addition of P_i does result in a decrease in fluorescence intensity (Figure 11). The decrease in fluorescence intensity follows the degree of phosphorylation measured using [^{32}P]P_i. At pH 7.0, the fluorescence change on addition of P_i is about half that observed at pH 6.4, although the level of phosphoenzyme formation is only reduced by ca. 20% (Figure 11). This suggests that the relationship between fluorescence intensity and extent of phosphorylation of P_i could be pH dependent. In contrast, the decrease in fluorescence intensity observed on addition of acetyl phosphate to DMC–ATPase (Figure 10) or ATP to either DMC–SR or DMC–ATPase is independent of pH (data not shown).

Discussion

We have studied labeling of the (Ca^{2+}–Mg^{2+})-ATPase with the apolar coumarin derivative 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC). As shown in Figure 1, this labels the (Ca^{2+}–Mg^{2+})-ATPase to a 1:1 molar ratio of label:ATPase. Thermolysin treatment of the labeled ATPase gave two labeled peptides corresponding to Leu_{342}–Ser_{345} and Leu_{342}–Cys_{344} (Table I) allowing the unambiguous assignment of the labeled residue as Cys-344.

Labeling of Cys-344 is consistent with the proposal of Bishop et al. (1988) that nonpolar sulfhydryl reagents will label Cys-344 and/or Cys-364 whereas polar sulfhydryl reagents will label Cys-670 and/or Cys-674. The same residue, Cys-344, has been labeled by Wakabayashi et al. (1990a) with NBD, but it gave a preparation which, although it could be phosphorylated, had almost no steady-state ATPase activity. In contrast, labeling with Br-DMC results in only a 25% decrease in the rate of ATP hydrolysis (Figure 1), and no effect

on the rate of hydrolysis of *p*-nitrophenyl phosphate (Figure 2) or on the Ca^{2+} or ATP dependence of ATP hydrolysis (data not shown) or on the maximal level of Ca^{2+} accumulation by SR vesicles. This would suggest that, although it is only 6 residues from the site of phosphorylation (Asp-351), Cys-344 is not directly involved in the function of the (Ca^{2+}–Mg^{2+})-ATPase. Cys-344 is not found to be conserved in other ATPase molecules. Thus, although the corresponding residue in the (Ca^{2+}–Mg^{2+})-ATPase of *Artemia* muscle is Cys (Palmero & Sastre, 1989), in the plasma membrane Ca^{2+} pump it is Asn (Shull & Greeb, 1988; Strehler et al., 1990) and in the (Na–K)-ATPase it is Ser (Rao et al., 1989).

Fluorescence responses of DMC–ATPase to pH, Ca^{2+}, and ATP are very different to those reported for the NBD-labeled ATPase. Thus, fluorescence of the NBD-labeled ATPase in the absence of Ca^{2+} is sensitive to pH and could be fitted to a titration curve with a pK value of 7.8 (Wakabayashi et al., 1990b). The pH dependence was similar to that observed previously for the ATPase labeled with fluorescein isothiocyanate, and attributed to pH-dependent changes in the E2–E1 equilibrium (Pick & Karlish, 1982; Froud & Lee, 1986a). As shown in Figure 5, the effect of pH on the fluorescence of DMC–ATPase fits to a model in which fluorescence intensity is sensitive to protonation of a residue with a pK of 6.8. This would be consistent with protonation of a His residue (Fersht, 1985). Similar titration curves are obtained in the presence of Ca^{2+} and Mg^{2+}, although the detailed interpretation of this data is more complex because of the effects of Ca^{2+} and Mg^{2+} on the fluorescence of DMC–ATPase described below. The pH response observed in the presence of Ca^{2+} indicates that the pH sensitivity of the fluorescence of DMC-ATPase cannot be attributed to changes in the E2–E1 equilibrium of the ATPase.

The His residues closest in the sequence to Cys-344 are His-190 in the transduction region of the ATPase and His-683 in the hinge region (Brandl et al., 1986). It has been shown that, in the presence of Ca^{2+}, the ATP analogue adenosine triphosphopyridoxal labels Lys-684, suggesting that in Ca_2E1 this region of the ATPase could be folded back to form part of the ATP binding domain, close to the residue that becomes phosphorylated, Asp-351 (Yamamoto et al., 1988). In the absence of Ca^{2+}, both Lys-492 and Lys-684 become labeled suggesting that these two residues are close together in the 3D structure of the ATPase and suggesting a conformation change in the presence of Ca^{2+} affecting this region of the ATPase (Yamamoto et al., 1989). Competitive monoclonal antibody binding studies have also shown that residues 510–515 and 662–666 in the proposed nucleotide binding domain and hinge domain of the ATPase are close together in the 3D structure (Tunwell et al., 1991).

As described below, effects of ligands on the fluorescence of DMC–ATPase can be understood in terms of three principal fluorescence states: high in the absence of ligands; intermediate in the presence of Mg or ADP; low in the presence of vanadate or AMP-PCP or following phosphorylation by P_i, ATP, or acetyl phosphate. A further distinction is that in the absence of Ca fluorescence changes on binding ligands are sensitive to protonation of the residue of pK 6.8, whereas in the presence of Ca^{2+} they are not. This change between Ca^{2+}-free and Ca^{2+}-bound forms could indicate a relocation of the residue of pK 6.8 relative to Cys-344, of the kind proposed by Yamamoto et al. (1989), described above.

Effects of Mg^{2+} on the (Ca^{2+}–Mg^{2+})-ATPase are complex. The true substrate for the ATPase is MgATP rather than ATP (Vianna, 1975) and the ATPase is only phosphorylated by P_i in the presence of Mg^{2+} (de Meis, 1981), with a binding constant for Mg^{2+} of 110 M^{-1} [see Froud and Lee (1986b)]. Mg^{2+} appears to bind competitively with Ca^{2+} at the Ca^{2+} binding sites on the ATPase (cytoplasmic on E1 and luminal on E2P) (Guillain et al., 1982; Froud & Lee, 1986a; Bishop & Al-Shawi, 1988); this binding is also competitive with binding of H^+ (Michelangeli et al., 1990a,b). Mg^{2+} also affects Ca^{2+} release from the unphosphorylated ATPase (Moutin & Dupont, 1991) and affects the rate of dephosphorylation of the phosphorylated ATPase (Wakabayashi et al., 1987). Moutin and Dupont (1991) have reported that release of Ca^{2+} from the ATPase, as monitored by changes in Trp fluorescence, is biphasic at pH 7.2 in the presence of Mg^{2+}, the relative amounts of the slow and fast phases of release varying with Mg^{2+} concentration, with the concentration of Mg^{2+} giving a half-value for this effect being about 5 mM. We have confirmed these results and find that the relative amounts of the two components fit to a simple binding equation for Mg^{2+} with a K_d of 4 mM (I. Henderson, J. M. East, and A. G. Lee, unpublished observations). The effect of Mg^{2+} on dephosphorylation of the ATPase at pH 7.0 reported by Wakabayashi et al. (1987) also fits to a K_d for Mg^{2+} of ca 4 mM.

As shown in Figure 7, addition of Mg^{2+} to DMC–SR in the presence of Ca^{2+} results in a change in fluorescence intensity, fitting to a single Mg^{2+} binding site of K_d 4 mM. Since the effect occurs in the presence of Ca^{2+}, it cannot be due to Mg^{2+} binding at the Ca^{2+} binding sites on E1 (Scheme I). The site for Mg^{2+} on the ATPase must be on the cytoplasmic side of the membrane since effects seen with DMC–SR and DMC–ATPase are identical (data not shown). The Mg^{2+} binding detected by changes in DMC–ATPase fluorescence must correspond to a third Mg^{2+} binding site on the ATPase (the other two being that at the Ca^{2+} binding site and that involved in phosphorylation of P_i). The identity of the K_d value obtained here with that affecting Ca^{2+} release from the ATPase and affecting dephosphorylation suggests that these effects might all correspond to binding to a single site, although this would need confirmation.

The effect of Mg^{2+} binding in the presence of Ca^{2+} is the same at pH 6 and 7, but in the absence of Ca^{2+}, the effect of binding at pH 7 is considerably less than at pH 6 (Figure 7). The dependence of fluorescence intensity on the concentration of Mg^{2+} still fits to a single binding site for Mg^{2+}, but with a shift in the apparent K_d value from 4.0 ± 0.54 mM at pH 6.0 to 1.78 ± 0.42 mM at pH 7.0. As shown in Figure 7 these changes can be simulated well making just two assumptions (i) only binding of Mg^{2+} to the E2 conformation results in a change in fluorescence intensity; and (ii) a change in fluorescence intensity is only seen for those forms of the ATPase protonated at the residue (pK 6.8) affecting the fluorescence of the DMC–ATPase, as described above (see Figure 5). The E2–E1 equilibrium for the ATPase was calculated as a function of pH and Mg^{2+}, largely as described in Froud and Lee (1986), but using the more accurate parameters obtainable from measurements of NBD–ATPase fluorescence, as described by Wakayabashi et al. (1990) (I. Henderson, J. M. East, and A. G. Lee, unpublished observations). With a true dissociation constant for Mg^{2+} of 4 mM (as for binding in the presence of Ca^{2+}), the experimental data can be fitted well (Figure 7). The simulations give apparent K_d values of 4.0 and 2.56 at pH 6 and 7, respectively, in reasonable agreement with the experimental values.

In the presence of Ca^{2+}, addition of either MgADP or ADP results in a decrease in fluorescence intensity, consistent with binding to a single site with K_d 9.5 ± 1.5 μM (Figure 9); the

effect is independent of pH (data not shown). This estimate is rather smaller than that from direct measurements of ADP binding, which fall in the range 15–25 μM (Meissner, 1973; Moller et al., 1980; Wakabayashi & Shigekawa, 1990). The magnitude of the fluorescence response to ADP is comparable to that seen with Mg^{2+}. Further, as for Mg^{2+}, in the absence of Ca^{2+}, addition of ADP or AMP-PCP at pH 7 results in a much smaller change in fluorescence intensity than at pH 6 (unpublished observations, but see Figure 10A), arguing for different conformational states in the presence and absence of Ca^{2+}. The effect of ADP on fluorescence is smaller in the presence of Mg^{2+} than in its absence, and the total change observed on addition of both Mg^{2+} and ADP is equal to that seen with either alone (compare Figures 7 and 9). The fluorescence response seen on addition of the nonhydrolyzable analogue of ATP, AMP-PCP, is larger than that seen with ADP, and it fits to a K_d of 21.1 ± 4.2 μM (Figure 9); direct binding measurements have given a value of 15 μM (Pang & Briggs, 1977).

Phosphorylation with P_i at pH 6.0 (Figure 6C) or acetyl phosphate (Figure 10D,E) or addition of vanadate (Figure 6D) results in a ca. 10% decrease in fluorescence intensity. A very similar change is observed on addition of ATP in the presence of Ca^{2+} (Figure 8). The initial decrease in fluorescence intensity observed on addition of ATP either could be due to phosphorylation or could follow directly from changes in the ATPase resulting from binding of ATP, if, as seems likely, binding ATP in the presence of Ca^{2+} had effects on fluorescence comparable to those seen with AMP-PCP. The steady state level of fluorescence seen in the presence of ATP can, however, be assigned to phosphorylated ATPase, since the ATPase is almost completely phosphorylated under these conditions (Michelangeli et al., 1990a,b).

Effects of phosphorylation and of binding Mg^{2+} or ADP to the ATPase are not additive. As shown in Figure 6, a greater fluorescence change is seen on addition of ATP in the presence of 1 mM Mg^{2+} than in the presence of 20 mM Mg^{2+}, and over a range of Mg^{2+} concentrations, the total change seen on addition of Mg^{2+} and then ATP is constant (data not shown), as described above for the effects of Mg^{2+} and ADP. The presence of ADP has no effect on the steady-state fluorescence level seen in the presence of ATP; thus the same steady-state level is observed following successive additions of ATP, or in the presence of an ATP-regenerating system to remove ADP (Figure 10). Direct addition of ADP following the addition of ATP also had no effect on the steady-state level of fluorescence (data not shown). Binding of Ca^{2+} to the luminal sites on the phosphorylated ATPase (to give Ca_2E2P) had no effect on the steady-state fluorescence level, since the same fluorescence changes were observed for the purified (Ca^{2+}–Mg^{2+})-ATPase in membrane fragments unable to a accumulate Ca^{2+} and in sealed SR vesicles, and since no changes in fluorescence were observed on addition of the Ca^{2+} ionophore BrA23187 to SR vesicles to discharge the accumulated Ca^{2+}. The conformational effect to which DMC–ATPase is sensitive would seem to be fully expressed in the phosphorylated form, and only partly expressed on binding Mg^{2+} or ADP to the nonphosphorylated ATPase.

As shown in Figure 11, the fluorescence changes observed on addition of P_i to the DMC–SR in the presence of Mg^{2+} and absence of Ca^{2+} at pH 6.4 follow the measured level of phosphorylation. At pH 7.0, the level of phosphorylation is about 80% of that observed at pH 6.4, in agreement with the measurements of Inesi et al. (1984) and our published model for phosphorylation (Froud & Lee, 1986b). The decrease in fluorescence intensity observed following phosphorylation at pH 7.0 is, however, only ca. 50% of that observed at pH 6.4 (Figure 11). The experimental data at pH 7.0 can be simulated if it is assumed that, as for binding of Mg^{2+}, a fluorescence change is observed on phosphorylation from P_i only if a site of pK 6.8 is protonated (Figure 11). If this is indeed the case, then it implies a difference in the conformational state of the ATPase following phosphorylation with P_i in the absence of Ca^{2+} and following phosphorylation in the presence of Ca^{2+} by acetyl phosphate (Figure 10) or ATP (data not shown) since in these cases fluorescence intensity is the same at pH 6.0 and 7.0. On the basis of experiments on the effects of unusual phospholipids on the function of the ATPase we suggested the possible presence of two phosphorylated forms of the ATPase E2′P and E2P:

$$Ca_2E2'P \rightleftharpoons E2'P \rightleftharpoons E2P \rightleftharpoons E2P_i \rightleftharpoons E2$$

with an equilibrium constant favoring the E2P form (Michelangeli et al., 1991). If the phosphorylated ATPase formed in the steady state from ATP and Ca^{2+} was predominantly in the E2′P form, this could account for the different pH dependencies of fluorescence described above. Stahl and Jencks (1987) suggested a conformational change on the ATPase following binding of ATP to Ca_2E1 relocating the nucleotide binding and phosphorylation domains on the ATPase, bringing the γ-phosphate of ATP close to Asp-351. We suggested that the proposed E2′P–E2P change could be the reverse of this process (Michelangeli et al., 1991).

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Registry No. ATPase, 9000-83-3; Cys, 52-90-4; ADP, 58-64-0; AMP-PCP, 3469-78-1; ATP, 56-65-5; Mg, 7439-95-4; Ca, 7440-70-2; vanadate, 14333-18-7; phosphate, 14265-44-2; 4-bromomethyl-6,7-dimethoxycoumarin, 88404-25-5; acetylphosphate, 590-54-5.

REFERENCES

Bishop, J. E., & Al-Shawi, M. K. (1988) *J. Biol. Chem. 263*, 1886–1892.

Bishop, J. E., Squier, T. C., Bigelow, D. J., & Inesi, G. (1988) *Biochemistry 27*, 5233–5240.

de Meis, L. (1981) in *The Sarcoplasmic Reticulum*, Wiley, New York.

de Meis, L., & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.

Dungee, W. (1977) *Anal. Chem. 49*, 442.

East, J. M., & Lee, A. G. (1982) *Biochemistry 21*, 4144–4151.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, W. H. Freeman, New York.

Froud, R. J., & Lee, A. G. (1986a) *Biochem. J. 237*, 197–206.

Froud, R. J., & Lee, A. G. (1986b) *Biochem. J. 237*, 207–215.

Guillain, F., Gingold, M. P., & Champeil, P. (1982) *J. Biol. Chem. 257*, 7366–7371.

Hardwicke, P. M., & Green, N. M. (1974) *Eur. J. Biochem. 42*, 183–193.

Hiratsuka, T. (1987) *J. Biochem. (Tokyo) 101*, 1457–1462.

Inesi, G., Lewis, D., & Murphy, A. J. (1984) *J. Biol. Chem. 259*, 996–1003.

Kawakita, M., & Yamashita, T. (1987) *J. Biochem. (Tokyo) 102*, 103–109.

Kubo, K., Suzuki, H., & Kanazawa, T. (1990) *Biochim. Biophys. Acta 1040*, 251–259.

MacLennan, D. H., Brandl. C. J., Korczak, B., & Green, N. M. (1985) *Nature 316*, 696–700.

Martin, D. W., & Tanford, C. (1981) *Biochemistry 20*, 4597–4602.
McWhirter, J. M., Gould, G. W., East, J. M., & Lee, A. G. (1987) *Biochem. J. 245*, 731–738.
Meissner, G. (1973) *Biochim. Biophys. Acta 298*, 906–926.
Michelangeli, F., Colyer, J., East, J. M., & Lee, A. G. (1990a) *Biochem. J. 267*, 423–429.
Michelangeli, F., Orlowski, S., Champeil, P., East, J. M., & Lee, A. G. (1990b) *Biochemistry 29*, 3091–3101.
Michelangeli, F., Grimes, E. A., East, J. M., & Lee, A. G. (1991) *Biochemistry 30*, 342–351.
Mitchinson, C., Wilderspin, A. F., Trinnaman, B. J., & Green, N. M. (1982) *FEBS Lett. 146*, 87–92.
Moller, J. V., Lind, K. E., & Andersen, J. P. (1980) *J. Biol. Chem. 255*, 1912–1920.
Moutin, M. J., & Dupont, Y. (1991) *J. Biol. Chem. 266*, 5580–5586.
Munkonge, F., East, J. M., & Lee, A. G. (1989) *Biochim. Biophys. Acta 979*, 113–120.
Murphy, A. J. (1976) *Biochemistry 15*, 4492–4496.
Obara, M., Suzuki, H., & Kanazawa, T. (1988) *J. Biol. Chem. 263*, 3690–3697.
Palmero, I., & Sastre, L. (1989) *J. Mol. Biol. 210*, 737–748.
Pang, D. C., & Briggs, F. N. (1977) *J. Biol. Chem. 252*, 3262–3266.
Pick, U. (1981a) *Eur. J. Biochem. 121*, 187–195.
Pick, U. (1981b) *FEBS Lett. 123*, 131–136.
Pick, U., & Karlish, S. J. (1980) *Biochim. Biophys. Acta 626*, 255–261.
Pick, U., & Karlish, S. J. (1982) *J. Biol. Chem. 257*, 6120–6126.
Rao, R., Nakamoto, R. K., & Slayman, C. W. (1989) in *Ion Transport* (Keeling, D., & Benham, C., Eds.) pp 35–54, Academic Press, London.
Rossi, B., de Assis, L. F., Gache, C., & Lazdunski, M. (1979) *J. Biol. Chem. 254*, 2302–2307.
Saito-Nakatsuka, K., Yamashita, T., Kubota, I., & Kawakita, M. (1987) *J. Biochem. (Tokyo) 101*, 365–376.
Shull, G. E., & Greeb, J. (1988) *J. Biol. Chem. 263*, 8646–8657.
Smith, R., & Martell, A. (1976) in *Critical Stability Constants*, Vol. 4, Plenum Press, New York.
Stahl, N., & Jencks, W. P. (1987) *Biochemistry 26*, 7654–7667.
Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T., & Carafoli, E. (1990) *J. Biol. Chem. 265*, 2835–2842.
Suzuki, H., Obara, M., Kuwayama, H., & Kanazawa, T. (1987) *J. Biol. Chem. 262*, 15448–15456.
Tunwell, R. E. A., Conlan, J. W., Matthews, I., East, J. M., & Lee, A. G. (1991) *Biochem. J. 279*, 203–212.
Vianna, A. L. (1975) *Biochim. Biophys. Acta 410*, 389–406.
Wakabayashi, S., & Shigekawa, M. (1990) *Biochemistry 29*, 7309–7318.
Wakabayashi, S., Ogurusu, T., & Shigekawa, M. (1987) *J. Biol. Chem. 262*, 9121–9129.
Wakabayashi, S., Imagawa, T., & Shigekawa, M. (1990a) *J. Biochem. (Tokyo) 107*, 563–571.
Wakabayashi, S., Ogurusu, T., & Shigekawa, M. (1990b) *Biochemistry 29*, 10613–10620.
Yamada, S., & Ikemoto, N. (1978) *J. Biol. Chem. 253*, 6801–6807.
Yamamoto, H., Tagaya, M., Fukui, T., & Kawakita, M. (1988) *J. Biochem. (Tokyo) 103*, 452–457.
Yamamoto, H., Imamura, Y., Tagaya, M., Fukui, T., & Kawakita, M. (1989) *J. Biochem. (Tokyo) 106*, 1121–1125.
Yamashita, T., & Kawakita, M. (1987) *J. Biochem. (Tokyo) 101*, 377–385.