Labeling the (Ca^{2+}–Mg^{2+})-ATPase of Sarcoplasmic Reticulum at Glu-439 with 5-(Bromomethyl)fluorescein†

H. I. Stefanova,‡ A. M. Mata,§ M. G. Gore,‡ J. M. East,‡ and A. G. Lee*,‡

Department of Biochemistry and SERC Centre for Molecular Recognition, University of Southampton, Southampton SO9 3TU, U.K., and Departamento de Bioquimica, Facultdad de Ciencias, 06080-Badajoz, Spain

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ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum was labeled with 5-(bromomethyl)fluorescein. A stoichiometry of one label per ATPase molecule was found, which was unaffected by the presence of ATP. Labeling resulted in a 60% decrease in ATPase activity. Sequencing identified the labeled residue as Glu-439. The fluorescence emission spectrum of the labeled ATPase was unaffected by the addition of Ca^{2+} or vanadate or by phosphorylation with either P_i or ATP. Measurement of the p*K* of the bound fluorescein and observation of quenching by KI were consistent with a relatively exposed location for the fluorophore. Measurements of fluorescence energy transfer located the position of Glu-439 relative to Lys-515 and Cys-344 and relative to the membrane surface. None of these distances changed on binding Ca^{2+} or vanadate.

Fluorescence probes attached to particular residues on a protein can be used to study conformational changes and distances to other labeled sites using the technique of fluorescence energy transfer. The difficulty lies in the attachment of a probe to a single residue on a protein. The (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum can be labeled specifically at Lys-515 with fluorescein isothiocyanate (FITC)[1] (Mitchinson et al., 1982) presumably because the fluorescein ring binds preferentially to the site on the ATPase normally occupied by adenosine. FITC has been found to label the corresponding lysyl residues in a number of other ATPases, although Xu (1989) has reported that a number of different lysyl residues are labeled in the (Na^+–K^+)-ATPase, all presumed to be located around the ATPase binding site. The ATPase contains 24 cysteinyl residues (MacLennan et al., 1985), and it has been reported that Cys-674 can be labeled specifically with iodoacetamide or 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonate (IAEDANS) (Suzuki et al., 1987; Yamashita & Kawakita, 1987) although it has also been reported that both Cys-670 and Cys-674 are labeled (Bishop et al., 1988). *N*-Ethylmaleimide has been found to label both Cys-344 and Cys-364 (Kawakita & Yamashita, 1987; Hara et al., 1987). Wakabayashi et al. (1990) have shown that it is possible to attach a fluorescence probe to Cys-344 alone by reacting 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 label.

One approach to maximize the chance of labeling at single sites on a protein is to use a probe of low general reactivity, so decreasing the extent of nonspecific labeling. We have explored the use of alkyl halides for labeling the ATPase. Although alkyl halides have been used predominantly for labeling the carboxyl groups of fatty acids in organic solvent (Haugland, 1992), they have also been shown to react with sulfhydryl groups (Hiratsuka, 1987). We have shown that 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) reacts specifically with Cys-344 on the ATPase. Here we show that 5-(bromomethyl)fluorescein (BrF) preferentially labels a single glutamyl residue, Glu-439, on the ATPase.

MATERIALS AND METHODS

5-(Bromomethyl)fluorescein (BrF) and 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) were obtained from Molecular Probes, eosin isothiocyanate (EITC) and rhodamine isothiocyanate (RITC) from Sigma, and phospholipids from Lipid Products or Avanti Polar Lipids. Dioleoylphosphatidylethanolamine (PE) was labeled with RITC using the protocol outlined by Fung and Stryer (1978) and was purified on preparative silica gel plates (Gutierrez-Merino et al., 1987). Sarcoplasmic reticulum (SR) and the purified (Ca^{2+}–Mg^{2+})-ATPase were prepared as described in East and Lee (1982). ATPase activities were determined at 25 °C by using a coupled enzyme assay in a medium 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. Maximum levels of phosphorylation of the ATPase were determined by incubating the ATPase (100 μg) in 0.5 mL of a medium containing 20 mM Hepes/Tris, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl, and 1 mM $CaCl_2$. The reaction was started by addition of 100 μM [γ-^{32}P]ATP and, after incubation at 25 °C for 10 s, was quenched by addition of 5 mL of an ice-cold mixture of 25% trichloroacetic acid in 0.13 M potassium phosphate. The quenched protein was collected by filtration through Whatman

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‡ University of Southhampton.

§ Facultdad de Ciencias.

[1] Abbreviations: Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin: BrF, 5-(bromomethyl)fluorescein; EITC, eosin isothiocyanate; FITC, fluorescein isothiocyanate; PE, dioleoylphosphatidylethanolamine; RITC, rhodamine isothiocyanate, SR, sarcoplasmic reticulum.

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GF/C glass fibre filters, washed, and counted in OptiPhase HighSafe 3. Concentrations of ATPase were estimated by using the extinction coefficient at 280 nm (1.2 L g^{-1} cm^{-1} for a solution in 1% SDS) given by Hardwicke and Green (1974).

Labeling Protocols. SR or purified ATPase was suspended to 10–12 mg/mL in buffer (40 mM Tris/MES, pH 7.0, 200 mM sucrose) and incubated with a 5-fold molar excess of BrF in the dark at 20 °C for 2 h; BrF was added from a 50 mM stock solution in dimethylformamide. Unbound BrF was separated from the labeled ATPase by centrifugation through two columns of Sephadex G-50 preequilibrated with the buffer used for labeling (Munkonge et al., 1989). Concentrations of ATPase were estimated from the absorbance at 280 nm in 1% SDS and KOH (5 mM) using the extinction coefficient given by Hardwicke and Green (1974) and the amount of bound fluorescein was estimated from the absorbance at 495 nm, using an extinction coefficient of 67 000 M^{-1} cm^{-1}.

SR was labeled with Br-DMC by incubation of Br-DMC and SR at a molar ratio of label to protein of 6:1 at room temperature in the dark, in 40 mM Tris/Mes (pH 7.5). Unbound label was removed by centrifugation through two columns of Sephadex G-50 (Stefanova et al., 1992). SR doubly labeled with BrF and Br-DMC was prepared by first incubating SR with BrF for 1 h followed by removal of unreacted BrF, addition of Br-DMC to a molar ratio of 6:1 (probe:protein), a further 1-h incubation, and final removal of unreacted probe as above.

ATPase was labeled with EITC by incubation of EITC and ATPase at various molar ratios up to 1:1 in 40 mM Tris/Mes (pH 8.0) for 45 min at room temperature, followed by removal of unreacted EITC as above. ATPase doubly labeled with BrF and EITC was prepared by first labeling with EITC and then with BrF as above. Concentrations of bound Br-DMC and EITC were determined following solubilization of samples in 1% SDS and KOH (0.1 M), using extinction coefficients of ϵ_{350} = 12 900 M^{-1} cm^{-1} (Stefanova et al., 1992) and ϵ_{500} = 83 000 M^{-1} cm^{-1} for Br-DMC and EITC, respectively.

Proteolysis of ATPase. Labeled ATPase (4 mg/mL) was incubated with trypsin (Sigma type XIII, TPCK-treated) in 10 mM Tris/HCl, pH 7.5, at a ratio of trypsin to ATPase of 1:20 (mg/mg). The digestion was terminated after 12 h by centrifugation at 14000*g* for 25 min. The supernatant was taken, and the pellet was washed with 100 μL of methanol and spun again at 14000*g* for 2.5 min. The two supernatants were combined and filtered through a 0.45-μm filter. The fluorescence of the labeled ATPase and that of the supernatant were recorded in 40 mM Tris/Mes (pH 8.0), using a Perkin Elmer LS-3B fluorometer, with excitation and emission wavelengths of 495 and 520 nm, respectively.

Peptides were separated using an Applied Biosystems Model 1783A gradient HPLC system equipped with a variable wavelength detector and an Applied Biosystems 980 fluorescence detector. Peptides were purified by reverse-phase chromatography using 25 x 0.4 cm octadecyl columns purchased from Jones Chromatography, using gradients made from 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, or from 10 mM aqueous potassium phosphate made to pH 7.7 with trifluoroacetic acid and a 1:9 (v/v) mixture of 10 mM aqueous potassium phosphate and acetonitrile. Absorbance of eluted peptides was determined at 235 nm, and fluorescence was excited at 495 nm and detected using a 540-nm cut-off filter. Fractions (1 mL) were collected from the HPLC column, and 100-μL aliquots were added to buffer (2.5 mL; 40 mM Tris/Mes, pH 8.0) for determination of fluorescence intensities.

Labeled ATPase was digested with thermolysin (Sigma, protease type X) for 3 h at room temperature with a ratio of thermolysin to ATPase of 1:50 (mg/mg), as described elsewhere (Stefanova et al., 1992). Peptides were purified as described above.

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

Reconstitution. Labeled ATPase was reconstituted with the desired amount of exogenous phospholipid (dioleoylphosphatidylcholine plus labeled phospholipid) by solubilization in cholate followed by dilution into buffer (East & Lee, 1982). Phospholipid (300 μmol) was mixed with buffer (40 μL; 50 mM potassium phosphate, pH 8.0, 1 M KCl, 0.2 M sucrose) containing $MgSO_4$ (5 mM), ATP (6 mM), and potassium cholate (83 μg) and sonicated to clarity in a bath sonicator (Megason). ATPase (40 μg) in a volume of 80 μL was then added and incubated for 1 h at room temperature. Samples were then diluted with 200 μL of buffer and stored on ice until use. For fluorescence measurements, samples (120 μL, equivalent to 15 μg of protein) were diluted into buffer (2.3 mL; 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 1 mM EGTA).

Fluorescence Measurements. Fluorescence spectra were recorded at 25 °C using an SLM-Aminco 8000C fluorometer. Samples contained 1 μM ATPase in 40 mM Tris/Mes and 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. A stock solution of KI (4.8 M) was prepared containing 1 mM sodium thiosulphate. All measurements were corrected for dilution.

For the ATPase labeled at single sites with donor and acceptor fluorophores, the efficiency of energy transfer (E) between donor and acceptor can be written as:

$$E = 1 - F/F_0 \quad (1)$$

where F and F_0 are fluorescence intensities of the donor in the presence and absence of acceptor, respectively. The efficiency of energy transfer is related to the distance between donor and acceptor (r) by

$$E = r^6/(r^6 + R_0^6) \quad (2)$$

where R_0 is the distance at which energy transfer is 50% efficient. Analysis of fluorescence energy transfer between fluorescein-labeled ATPase and RITC-PE needs to take into account the distribution of fluorescence acceptors in the plane of the membrane. In other papers we have adopted three approaches to the analysis of the problem (Gutierrez-Merino et al., 1987; Munkonge et al., 1989). In the first, the ATPase is considered as a cylinder embedded in the phospholipid bilayer, and the position of the label on the ATPase is characterized by its height h above the phospholipid-water interface (defined as the location of the label in the headgroup of PE) and the distance d between the site on the ATPase and the circumference of the protein (Gutierrez-Merino, 1981; Gutierrez-Merino et al., 1987). The two other approaches due to Koppel et al. (1979) and Dewey and Hammes (1980) consider RITC-PE as being randomly distributed on the surface of a plane a distance h below the position of the donor on the protein.

The value of R_o is given by

$$R_o = (9.79 \times 10^3)(\kappa^2 n^{-4} Q J)^{1/6} \quad (3)$$

where κ^2 is the orientation factor, n is the dielectric constant, Q is the quantum yield of the donor in the absence of acceptor, and J is the spectral overlap integral. We determined the quantum yield at pH 7.2 for BrF-labeled ATPase to be 0.277, relative to a value of 0.9 for free fluorescein in NaOH (Bridges, 1981); the quantum yield of the bound fluorescein was very dependent on pH, and, for example, had a value of 0.56 in 1 M KOH. The spectral overlap integral between BrF-labeled ATPase and RITC-PE was determined to be 2.973 x 10^{-13} cm^6/mol and for the ATPase double labeled with Br-DMC and BrF to be 1.14 x 10^{-13} cm^6/mol, both at pH 7.2. With a value of n of 1.33 (Gutierrez-Merino et al., 1987), and assuming a value for the orientation factor κ^2 of 2/3, values of R_o (Å) of 49.7 and 40.6 are obtained for the BrF/EITC and Br-DMC/BrF pairs, respectively, at pH 7.2.

Fluorescence polarizations for ATPase labeled with BrF and EITC have been measured as 0.26 and 0.35 respectively at 25 °C. The calculations of Dale et al. (1979) [see also Dos Remedios et al. (1987)] would then suggest that the error in distance measurements for the double labeled ATPase assuming the value of κ^2 of 2/3 will be ca. ±10%. As described elsewhere, errors in measurements of distances from sites on the ATPase to the phospholipid bilayer caused by assuming a value of κ^2 of $^2/_3$ will be even less, because of the random orientation of lipid-bound acceptor groups in the plane of the membrane (Munkonge et al., 1989).

Fluorescence energy transfer experiments for the ATPase doubly labeled with BrF and EITC were analyzed both by measuring fluorescence intensities at 520 nm and by curve fitting. The emision spectra of the doubly labeled systems (BrF and EITC labeled) were fitted to linear sums of the emission spectra of BrF-labeled ATPase and EITC-labeled ATPase, using the procedure described in Munkonge et al. (1988). Identical results were obtained using either approach.

RESULTS

Labeling of the (Ca^{2+}–Mg^{2+})-ATPase by BrF. When the purified (Ca^{2+}–Mg^{2+})-ATPase was incubated with a 5-fold molar excess of BrF at pH 7.0, 20 °C, for up to 2 h, fluorescein label was incorporated into the ATPase up to 7.3 nmol of label/mg of protein, equivalent to a molar ratio of label to ATPase of 0.84:1, calculated assuming pure ATPase with a molecular mass of 115 kDa. The extent of incorporation increases monoexponentially with time, described by a rate constant of 0.02 min^{-1} and a maximum labeling ratio of 8.2 nmol of label/mg of protein equivalent to a molar ratio of label to ATPase of 0.94:1 (Figure 1). The steady-state ATPase activity of the ATPase decreases in parallel with the incorporation of label; the data fit to a single-exponential process again with a rate constant of 0.02 min^{-1}, with a maximal level of inhibition of 59% (Figure 1). Addition of a further 5-fold excess of label after 1 h had no effect on the final level of labeling. Labeling intact SR vesicles gives a slightly lower maximum level of incorporation (7.6 nmol of label/mg of protein or a molar ratio of label to ATPase of 0.88:1) but the same rate constant for labeling: the presence of 2.5 mM ATP and 5 mM Mg^{2+} during the labeling reaction had no effect on the level of incorporation of label (Figure 1).

Identification of the Residue Labeled by BrF. Following labeling with excess BrF and removal of unreacted BrF, the ATPase was digested with thermolysin, and soluble peptides were separated from membranous fragments by centrifugation. As estimated by fluorescence measurements, more than 90% of the initial bound fluorescein was recovered in the supernatant. The peptides in the supernatant were analyzed by reverse-phase HPLC with an acetonitrile gradient in 0.1% trifluoroacetic acid. As shown in Figure 2, fluorescence is observed in five major bands and a number of minor bands. In separate experiments it was shown that unreacted BrF runs at the position of peak 2 in Figure 2. Peaks 1, 3, 4, and 5 correspond to 14%, 13%, 32%, and 10% of the total bound fluorescence, respectively. The major peaks were further purified by reverse-phase HPLC with an acetonitrile gradient in 10 mM potassium phosphate (pH 7.7) (Figure 3). As shown, the major peak (peak 4 in Figure 2) gave one major peak (Figure 3B) on the second column. The peptide responsible for this peak was sequenced to give VG, possibly followed by a nonstandard residue running with diphenylthiourea. Ther-

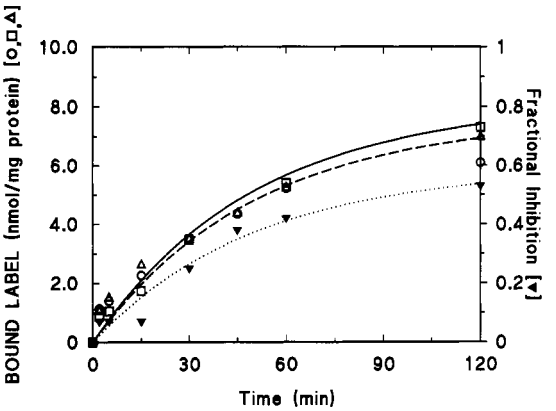

FIGURE 1: Modification of the ATPase by BrF. The (Ca^{2+}–Mg^{2+})-ATPase (□) or SR (○, △) (6 mg) was incubated with BrF at a probe to protein molar ratio of 5:1 in 2 mL of 40 mM Tris/Mes (pH 7.0) at 20 °C in the absence (□, ○) or presence (△) of 2.5 mM ATP and 5 mM Mg^{2+}. After the given times, 250-μL aliquots were taken, and unreacted BrF was separated from labeled protein on Sephadex G-50. The ratio of bound label to ATPase was estimated by absorbance. ATPase activities for the purified ATPase (▼) were measured at pH 7.2, 100 mM KCl, 25 °C, 2.1 mM ATP, and maximally stimulating concentrations of Ca^{2+}, as described under Materials and Methods, and are expressed as fractional inhibition, based on an initial ATPase activity for the unmodified ATPase of 3.0 IU/mg of protein. The solid and broken lines show fits of the labeling data to first-order processes with a rate constant of 0.02 min^{-1} and final labeling ratios of 0.94 and 0.88 for ATPase and SR, respectively. The dotted line shows a fit of the inhibition data to a first-order process with a rate constant of 0.02 min^{-1} and a final level of inhibition of 59%.

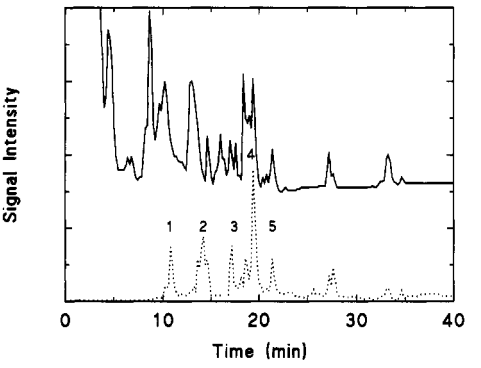

FIGURE 2: Analysis of peptide fragments generated by thermolysin treatment. Soluble peptides obtained by treatment of labeled ATPase with thermolysin were fractionated by reverse-phase HPLC on a C_{18} column at a flow rate of 1 mL/min. The peptides were eluted with a linear gradient of acetonitrile/0.1% TFA in water/0.1% TFA from 20% to 60% acetonitrile. The top and bottom traces show absorbance and fluorescence emission, respectively.

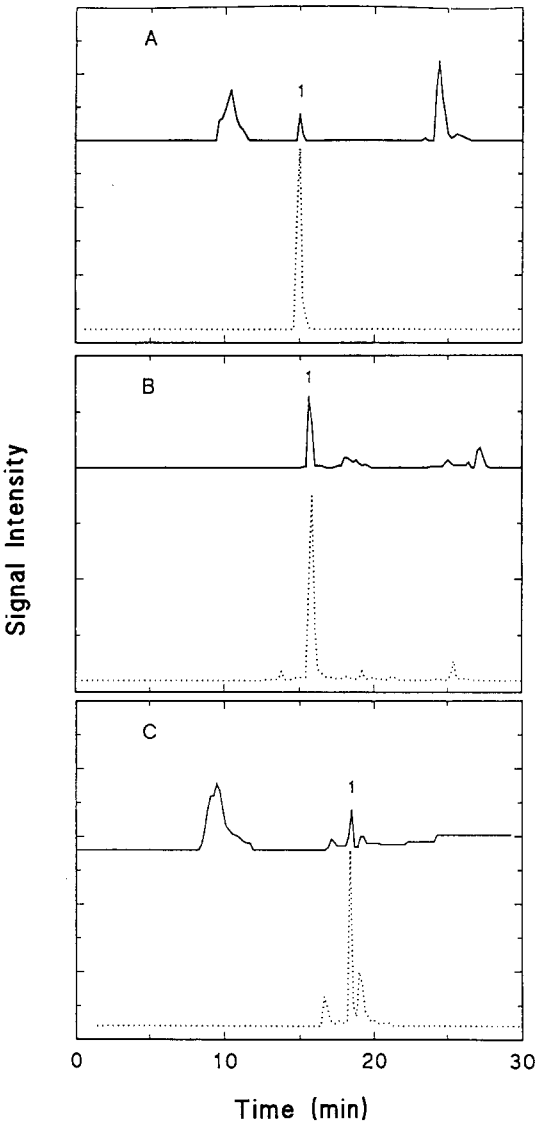

FIGURE 3: Purification of peptide fragments generated by thermolysin treatment. Peaks 1 (A), 4 (B), and 5 (C) obtained as shown in Figure 2 by thermolysin treatment of the labeled ATPase were further purified on a C_{18} column at a flow rate of 1 mL/min, with a linear gradient of acetonitrile in 10 mM potassium phosphate (pH 7.7) from 0% to 30% acetonitrile. The top and bottom traces show absorbance and fluorescence, respectively.

molysin cleaves on the N-terminal side of hydrophobic amino acids (Croft, 1980), and the (Ca^{2+}–Mg^{2+})-ATPase contains the unique sequence $K_{436}V_{437}G_{438}E_{439}A_{440}$ suggesting modification of Glu-439 by Br-F. Peaks 1 and 5 in Figure 2 also gave single major peaks on the second column (Figure 3), but these gave no sequence.

In order to obtain larger peptide fragments for sequencing, the labeled ATPase was also cleaved with trypsin (Figure 4). Of the initial fluorescence, 88% was obtained in soluble peptides. The soluble peptides were fractionated on a C_{18} column, and a large number of fluorescent peaks were obtained, of which one (peak 3) corresponds to free BrF (Figure 4). Fractions (1 mL) were collected from the HPLC column, and the fluorescence of each fraction was determined. Peaks 1, 2, 4, 5, 6, 7, and 8 contain 2%, 8%, 7%, 6%, 6%, 8%, and 11%, respectively, of the fluorescence recovered from the first column. The result of further purification of the major peaks using an acetonitrile gradient in 10 mM potassium phosphate is shown in Figure 5. The largest peak from the first column (peak 8) was found to contain a single peptide (Figure 5D)

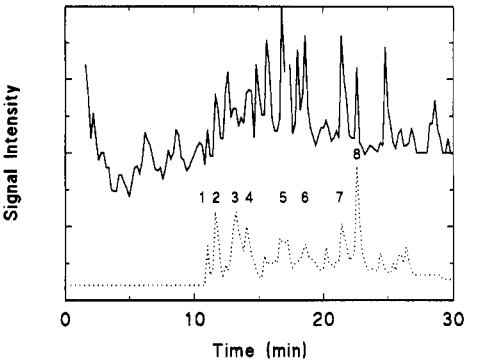

FIGURE 4: Analysis of peptide fragments generated by trypsin treatment. Soluble peptides obtained by treatment of labeled ATPase with trypsin were fractionated by reverse-phase HPLC on a C_{18} column at a flow rate of 1 mL/min. The peptides were eluted with a linear gradient of acetonitrile/0.1% TFA in water/0.1% TFA from 20% to 50% acetonitrile. The top and bottom traces show absorbance and fluorescence emission, respectively.

which, on sequencing, gave VG-ATETALTTLVEK: comparison with the published sequence for the (Ca^{2+}–Mg^{2+})-ATPase identifies the expected amino acid at position 3 of this peptide as Glu-439 (Table I). At the third cycle of Edman degradation the yield of PTH-Glu released was only approximately 6% of that expected from the data shown in Figure 6. This large decrease in yield at cycle 3 is not typical of glutamyl residues under these sequencing conditions since the Glu at cycle 6 is found in the expected yield and that in cycle 14 is at a comparable level even though the remaining peptide chain was being washed off from the glass fiber disc in the sequencer. The HPLC analysis of PTH derivatives released at cycle 3 failed to show a new peak corresponding to a derivative of Glu. However, it is probable that the hydrophobic nature of the fluorescein-modified side chain would result in the PTH derivative eluting after the end of the normal HPLC gradient or possibly under diphenylthiourea.

As shown, peak 2 from the first column (Figure 4) gave two major fluorescent bands on the second column (Figure 5A). On sequencing, both gave an unidentifiable first residue and then sequences SPAK and LMK, respectively, for peaks 1 and 2. The ATPase contains sequences CSPAK and QLMK from which these peptides are presumably derived. The first of these peptides contains a Cys residue that could have been labeled by BrF, but no evidence was found for the elution of such a labeled amino acid from the reverse-phase column; the second peptide contains no residue likely to have reacted with BrF. Free BrF run under these conditions gives two major peaks eluting at ca. 22% and 19% acetonitrile (the positions of peaks 1 and 2) so that the peaks shown in Figure 4A could correspond to peptides co-eluting with either free BrF or a hydrolysis product of BrF. Peak 4 from the first column was found to contain a complex mixture of peptides (Figure 5B), the most fluorescent of which (peak 1 in Figure 5B) was sequenced (Figure 6) to give the peptide containing Glu-439 shown in Table I. Peak 7 from the first column gave two major fluorescent peaks on the second column (Figure 5C); sequencing of the most fluorescent of the peaks (peak 1) again gave a peptide containing Glu-439 as shown in Figure 6, whereas the minor peak gave the sequence DIVPGDIVE, containing a glutamyl reside as a possible candidate for labeling (Table I). For proteolysis with trypsin therefore, ca. 25% of the bound fluorescein has been identified in peptides containing Glu-439. Since this was also the only labeled residue identified after proteolysis with thermolysin, we suggest that this is the major site of labeling of the ATPase.

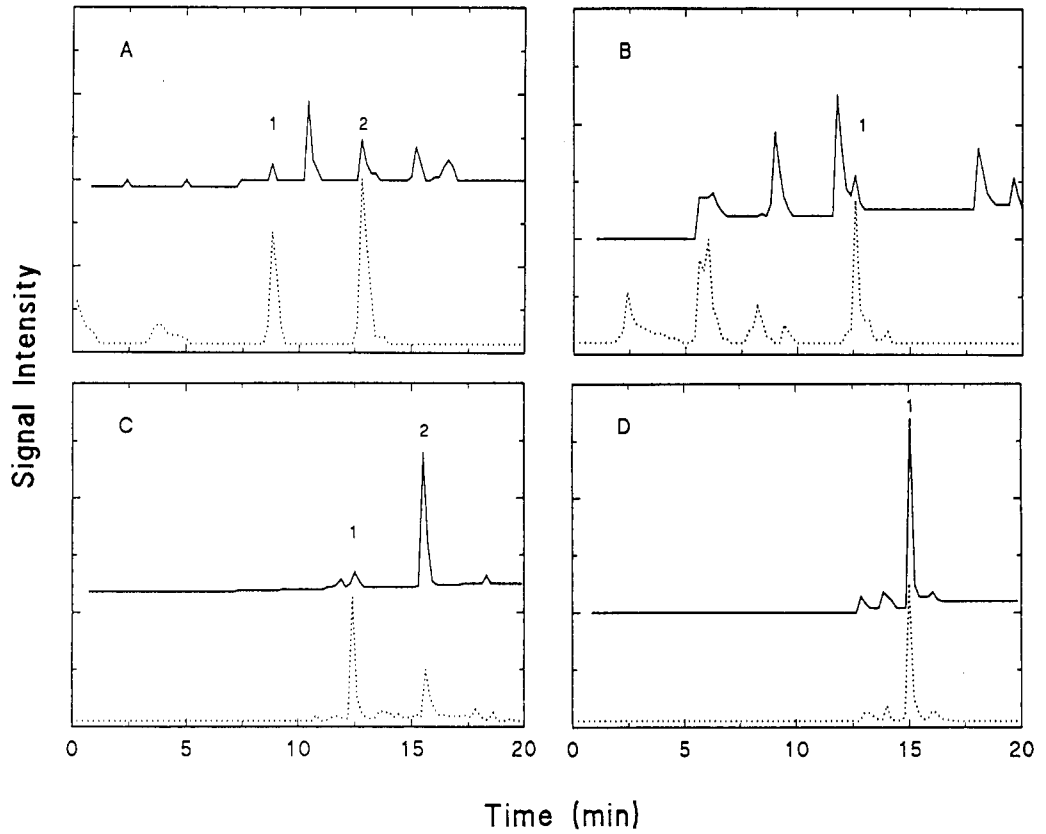

FIGURE 5: Purification of peptide fragments generated by trypsin treatment. Peaks 2 (A), 4 (B), 7 (C), and 8 (D) obtained as shown in Figure 4 by trypsin treatment of the labeled ATPase were further purified on a C_{18} column at a flow rate of 1 mL/min, with a linear gradient of acetonitrile in 10 mM potassium phosphate (pH 7.7) from 10% to 30% acetonitrile. The top and bottom traces show absorbance and fluorescence, respectively.

Table I: Sequences of Purified Labeled Peptides

proteolysis	peptide peak[a]	amino acid sequence and assignment[b]
thermolysin	4	$V^{437}GX$
trypsin	4	$V^{437}GE^{c}ATETAL$
	7	$V^{437}GXATETALTTL$
		$D^{144}IVPGDIVE$
	8	$V^{437}GXATETALTTLVEK$

[a] Peaks from the first HPLC purifications of proteolytic fragments of the labeled ATPase (Figures 2 and 4) were further purified on a second column as shown in Figures 3 and 5 and sequenced. [b] X represents the position of a gap in the sequence or possibly a residue eluting from the reverse-phase column in the same position as diphenylthiourea. Examination of the published sequence of the ATPase (MacLennan et al., 1985) shows that the position of this residue corresponds to Glu-439. [c] Sequencing shows an almost normal recovery of PTH amino acid derivative for this residue in the major fluorescent component of peptide peak 4 (Figure 6).

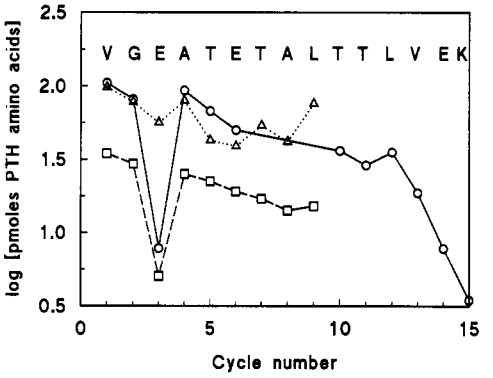

FIGURE 6: Recovery of PTH derivatives of amino acids released in sequencing analysis of BrF-labeled peptides obtained by treatment with trypsin. Components are labeled as in Figure 4 and Table I: peak 8 (○); major fluorescent components of peak 7 (□); and peak 4 (△).

Fluorescence Properties of BrF Labeled ATPase. The (Ca^{2+}–Mg^{2+})-ATPase labeled with BrF exhibits fluorescence centered at 520 nm with an excitation maximum at 495 nm. The fluorescence intensity of the labeled ATPase is pH dependent (Figure 7), and a pH titration gives a pK value of 6.98 ± 0.07, compared to values of 6.83 ± 0.04 and 6.46 ± 0.09, respectively, for BrF and free fluorescein in buffer (Figure 7). The fluorescence data fit to relative fluorescence intensities of the dianion and monoanion species of 4.3 and 3.6 for BrF-ATPase and free BrF, respectively, compared to a value of 29.2 for fluorescein.

To test the accessibility of the fluorescein label on the ATPase, we studied quenching of BrF-ATPase fluorescence by iodide. In the simplest case of dynamic quenching, fluorescence quenching fits to the Stern–Volmer relationship:

$$F_o/F = 1 + K_{SV}[Q]$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the quencher concentration, and K_{SV} is a constant. As shown in Figure 8, quenching of free BrF and fluorescein by KI fits well to the Stern–Volmer equation with K_{SV} values of 7.4 and 6.9 M^{-1}, respectively. In contrast, quenching of BrF-ATPase is less efficient, with a K_{SV} value of 2.7 M^{-1}.

The marked pH dependence of fluorescence intensity (Figure 7) makes it difficult to study effects of ligands on fluorescence. Nevertheless, no significant changes in fluorescence intensity (<2%) were observed on addition of Ca^{2+}, Mg^{2+}, or vanadate to the labeled ATPase. Similarly, addition of P_i in the presence of Mg^{2+} and EGTA at pH 6.0 (conditions

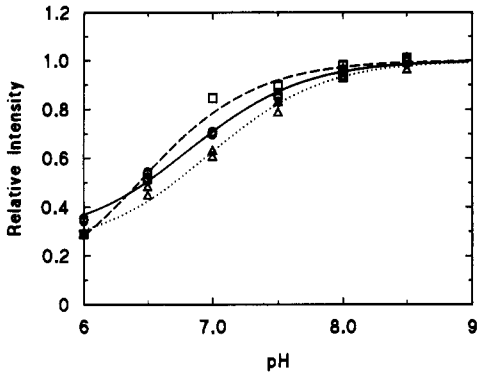

FIGURE 7: Effect of pH on the fluorescence intensity of free BrF (○) and fluorescein (□) and of BrF-ATPase labeled to a 1:1 molar ratio (△). The lines show nonlinear least-squares fits to the Henderson–Hasselbach equation with pK values of 6.83 for BrF (solid line), 6.46 for fluorescein (broken line), and 6.98 for BrF-ATPase (dotted line), with relative fluorescences for dianion and monoanion forms as described in the text.

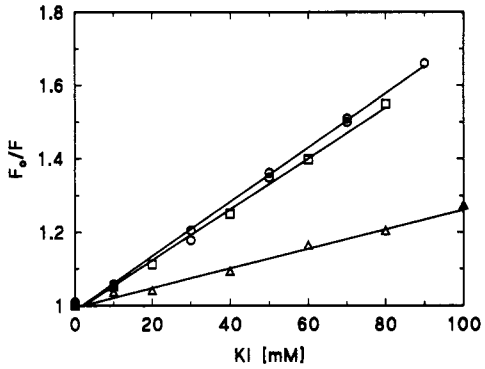

FIGURE 8: Stern–Volmer plot of F_o/F against the concentration of added KI for (○) BrF, (□) fluorescein, and (△) BrF-ATPase in 50 mM Hepes/KOH (pH 7.0). The solid lines show linear least-squares fits to the Stern–Volmer equation.

likely to lead to the phosphorylation of the ATPase) or addition of ATP in the presence of Mg^{2+} and Ca^{2+} resulted in no significant change in intensity (data not shown).

The fluorescence emission spectrum of fluorescein overlaps the absorption spectra of eosin and rhodamine so that fluorescence energy transfer experiments are possible between BrF-ATPase and these latter two probes. It has been shown that, at molar ratios of probe to protein of less than 1:1, EITC labels the ATPase selectively at Lys-515 (Papp et al., 1987). Figure 9 compares the fluorescence emission spectrum of the ATPase labeled to a molar ratio of bound BrF to ATPase of 0.69:1 with that of the ATPase double labeled with BrF and FITC at molar ratios of bound BrF to ATPase and bound EITC to ATPase of 0.48:1 and 0.65:1, respectively, scaled to equal concentrations of bound BrF. As shown, ATPase labeled with EITC alone shows no significant fluorescence intensity at 520 nm, so that the intensity at 520 nm can be used to measure the efficiency of energy transfer between fluorescein and eosin labels. Alternatively, the spectrum of the doubly labeled ATPase can be fitted to a sum of two component spectra, with identical results (Figure 9, Table II). Table II shows the efficiency of transfer measured at three different EITC labeling ratios and the efficiency calculated from these data for a 1:1 molar ratio of bound EITC to ATPase. Table II also shows the calculated values for the distance of separation between bound fluorescein and eosin labels. The efficiency of transfer is unaffected by addition of Ca^{2+} or vanadate in the presence of Mg^{2+}; it is also unaffected by addition of the detergent $C_{12}E_8$ at 2.5 mg/mL (data not shown).

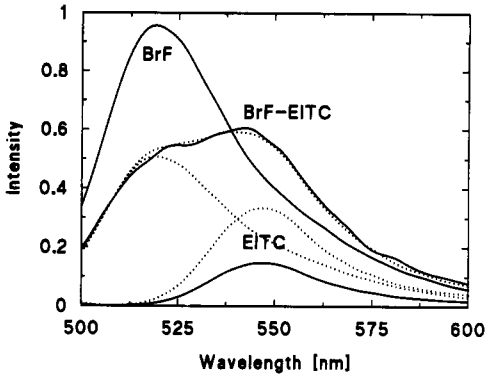

FIGURE 9: Fluorescence emission spectra showing energy transfer from fluorescein to eosin on the doubly-labeled ATPase. Shown (solid lines) are emission spectra for the ATPase labeled with BrF (0.035 μM ATPase; molar ratio of BrF to ATPase of 0.69:1) and EITC (0.045 μM ATPase; molar ratio of EITC to ATPase of 0.62:1) alone and the double-labeled preparation (0.043 μM ATPase; molar ratios of BrF and EITC to ATPase of 0.48 and 0.65:1, respectively), at equal concentrations of fluorescein label (BrF and BrF-EITC) and eosin label (BrF-EITC and EITC). The dotted lines show the best fit of the spectrum of the doubly labeled ATPase to the weighted sum of the spectra of the singly labeled species and the two components of the best fit, with weighting factors of 0.55 and 2.27, respectively, for BrF-labeled and EITC-labeled ATPase; a weighting factor of 0.55 for BrF-labeled ATPase corresponds to an efficiency of energy transfer of 0.45 (Table II).

Table II: Energy Transfer between Bound BrF and EITC Labels

molar ratio of EITC to ATPase	measured E^a	calculated E for 1:1 labeling	r (Å)^b
0.28^c	0.19	0.69	43
0.39^d	0.3	0.77	41
0.65^e	0.45	0.69	43

^{a} Efficiency of transfer E calculated as $(1 - F/F_o)$ where F_o and F are fluorescence intensities for BrF-labeled ATPase and double-labeled ATPase, respectively, normalized to equal concentrations of bound BrF label. ^{b} Calculated with an R_o value of 49.7 Å. ^{c} Molar ratio of bound BrF to ATPase of 0.61:1. ^{d} Molar ratio of bound BrF to ATPase of 0.53:1. ^{e} Molar ratio of bound BrF to ATPase of 0.48:1.

Table III: Energy Transfer between Bound BrC and BrF Labels^a

conditions	F/F_o^{b}	r (Å)^c
20 μM Ca^{2+}	0.47	39.8
1 mM EGTA + 5 mM Mg^{2+}	0.46	39.6
1 mM EGTA + 5 mM Mg^{2+} + 100 μM vanadate	0.46	39.6

^{a} The ATPase was labeled with Br-DMC and BrF to 1:1 molar ratios, and the fluorescence intensities for the bound coumarin label were determined in 50 mM Hepes/KOH (pH 7.0) in the presence of the given ligands. ^{b} F_o and F are, respectively, the fluorescence intensities measured at 425 nm for the ATPase labeled with Br-DMC alone and doubly labeled with Br-DMC and BrF. ^{c} Calculated with R_o = 40.6 Å.

We have shown that Br-DMC labels the ATPase at Cys-344 (Stefanova et al., 1992). Efficient energy transfer is observed between coumarin and fluorescein labels on the ATPase for the ATPase doubly labeled with Br-DMC and BrF (Table III); the efficiency of transfer is unaffected by addition of Ca^{2+} or vanadate in the presence of Mg^{2+} (Table III) or by addition of $C_{12}E_8$ at concentrations up to 2.5 mg/mL (data not shown).

Figure 10 shows the fluorescence emission spectrum of the ATPase labeled with BrF and reconstituted into bilayers of dioleoylphosphatidylcholine and RITC-PE, at a phospholipid to protein molar ratio of 300:1. As shown, increasing the concentration of RITC-PE in the bilayer results in a decrease in the intensity of fluorescein fluorescence. Increasing the

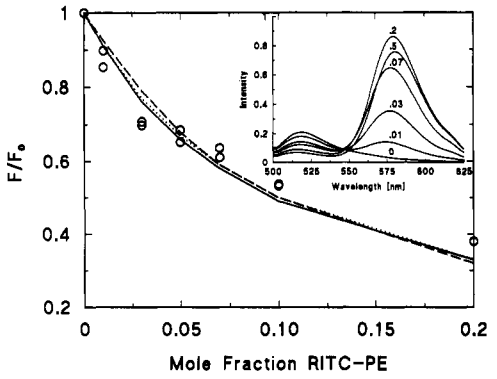

FIGURE 10: Fluorescence quenching (F/F_0) of BrF-labeled ATPase reconstituted into phospholipid mixtures as a function of the mole fraction of FITC-PE, at a molar ratio of phospholipid to ATPase of 300:1. The lines are theoretical calculations, using (solid line) the approach of Dewey and Hammes (1980) with h = 70 Å, (dashed line) the apprach of Koppel et al. (1979) with h = 68 Å, and (dotted line) the approach of Gutierrez-Merino (Gutierrez-Merino, 1981; Gutierrez-Merino et al., 1987) with d = 5 Å and h = 72 Å. The inset shows the fluorescence emission spectra at the given mole fractions of RITC-PE.

molar ratio of RITC-PE above 0.2 results in a decrease in fluorescence intensity for the RITC-PE (Figure 10), presumably reflecting a change in the environment of the rhodamine group in the bilayer at high molar ratios of label. Energy transfer data are therefore only analyzed up to a molar ratio of 0.2 RITC-PE (Figure 10).

DISCUSSION

The chemical reactivity of carboxylic acids in water is low, so that only very few reagents are known that will modify carboxyl groups in proteins. Reaction is possible with carbodiimides, and it has been shown that hydrophobic carbodiimides modify carboxyl groups in the transmembrane region of the (Ca^{2+}–Mg^{2+})-ATPase (Pick & Racker, 1979; Chadwick & Thomas, 1984; Munkonge et al., 1989; Mata et al., 1993). Alkyl halides react with the carboxyl groups of fatty acids in organic solution, but their higher intrinsic reactivity for sulfhydryl groups means that they generally label cysteinyl residues in proteins (Haugland, 1992) although there are examples of labeling glutamyl residues in proteins (Takahashi et al., 1967; Hartman et al., 1975). Haloacetates have been shown to react with a single glutamyl residue in ribonuclease T_1; since labeling was only observed with anionic alkylating agents, it is assumed that the site of labeling is one of high positive overall charge (Takahashi et al., 1967). 3-Haloacetol phosphates and sulfates have been shown to modify a single glutamyl residue in the active site of triosephosphate isomerase, and it is assumed that this glutamyl residue has an unusually high nucleophilicity (Hartman et al., 1975).

The low reactivity of the carboxyl group makes it an attractive candidate for specific labeling: the chances of nonspecific labeling are low. We have therefore explored the reaction of a variety of fluorescent alkyl halides with the (Ca^{2+}–Mg^{2+})-ATPase. We have shown that 4-(bromomethyl)-6,7-dimethoxycoumarin reacts not with carboxyl groups on the ATPase but rather with Cys-344 (Stefanova et al., 1992). Here we have studied labeling with 5-(bromomethyl)fluorescein (Br-F). As shown in Figure 1, this labels the ATPase to ca. 8 nmol of label/mg of protein, corresponding to a 1:1 molar ratio of label to ATPase. The rates of labeling of purified ATPase (present as membrane fragments) and of sealed vesicles of sarcoplasmic reticulum are the same (Figure 1), suggesting that the site modified on the ATPase is on the cytoplasmic side of the membrane. Both the rate of labeling and the rate of inhibition of ATPase activity fit to simple first-order kinetics with the same rate constant of 0.02 min^{-1}, consistent with labeling a single site on the ATPase with the reduction in activity being proportional to the extent of labeling. The rate of labeling is unaffected by the presence of ATP (Figure 1), suggesting that the site of labeling is not in the ATP binding site. In this respect, labeling with BrF is unlike labeling with fluorescein isothiocyanate which is competitive with binding of ATP (Pick, 1981a). The level of inhibition of ATPase activity calculated at a 1:1 labeling ratio (Figure 1) is 59%. Incomplete inhibition of activity suggests that the label is not located at one of the essential binding sites on the ATPase (for Ca^{2+} or ATP). Partial inhibition of ATPase activity was also observed following labeling of the ATPase at Cys-344 with Br-DMC (Stefanova et al., 1992) and merely indicates a decrease in rate for at least one of the steps in the reaction sequence.

Levels of labeling of the ATPase are expressed in terms of total protein estimated spectroscopically using the extinction coefficient given by Hardwicke and Green (1974). Polyacrylamide gels of our preparations of the purified ATPase stained with Coomassie Blue suggest that the ATPase is essentially pure (>97%) (Gould et al., 1987). However, the maximum level of phosphorylation we observed for the preparations of ATPase used here was 3.5 nmol/mg of protein, corresponding to 40% of that expected for a pure protein of molecular weight 115 000; similar observations have been made by others [see Starling et al. (1993)]. The reason for the lower than expected levels of phosphorylation have not been established, but it clearly does not imply a low purity for the preparation. Thus, for example, labeling with FITC is known to be highly specific for Lys-515 on the ATPase, and the maximam labeling ratio observed with FITC is one FITC per ATPase molecule, calculated assuming that the ATPase sample is pure (Mitchinson et al., 1982). Similarly, Br-DMC has been found to be specific for Cys-344 on the ATPase, and again a maximum labeling ratio of one coumarin per ATPase was found, calculated assuming that all the protein in the sample was (Ca^{2+}–Mg^{2+})-ATPase (Stefanova et al., 1992).

Thermolysin treatment of the labeled ATPase released essentially all (90%) of the label into the supernatant. HPLC purification of the released peptides and sequencing gave a peptide VGX, identifying the labeled residue as Glu-439 (Table I). This peptide contained 32% of the initial bound fluorescence; attempts to sequence other fluorescent peaks obtained by HPLC separation gave no identifiable amino acids. Glutamyl esters are expected to be relatively unstable under the conditions of proteolysis and sequencing (De La Mare et al., 1972), so that the yield of labeled peptide found here is relatively high and must represent the major site of labeling. In particular, no evidence was found for the presence of labeled cysteinyl residues, although these were readily detected following labeling with 4-(bromomethyl)-6,7-dimethoxycoumarin (Stefanova et al., 1992).

To further characterize the site of labeling, we studied proteolysis with trypsin (Figures 4 and 5). The most fluorescent peptide obtained following proteolysis again gave the site of labeling as Glu-439 (Table I). This same labeled residue was identified in two other peptides (Table I) and in all accounted for 25% of the bound fluorescein label. Of the other fluorescent peaks obtained after the first HPLC purification, one was found on a second column to contain two peptides (Figure 5A), one of which (LMK) contained no

residues that could have been labeled by BrF, and the other (SPAK), although containing a cysteinyl residue that might have reacted, failed, upon sequencing, to show the presence of a modified cysteinyl residue; it is likely that these peptides co-eluted with unreacted or hydrolyzed BrF, which in separate experiments was shown to elute at the same position as these two peptides. A peptide DIVPGDIVE was also obtained (Figure 5C), containing ca. 2% of the total bound fluorescence (Table I). This could indicate minor labeling of Glu-152 in the ATPase.

Measurements of labeling stoichiometry and time courses of labeling and inhibition of ATPase activity indicate labeling of a single residue on the ATPase. Sequencing of thermolysin- and trypsin-generated fragments of the ATPase give Glu-439 as the site of labeling. This residue is part of the postulated phosphorylation domain of the ATPase (Brandl et al., 1986). Although it is conserved in all the known sarcoplasmic reticulum/endoplasmic reticulum (Ca^{2+}–Mg^{2+})-ATPases (Brandl et al., 1986; Palmero & Sastre, 1989; Magyar & Varadi, 1990), in the plasma membrane (Ca^{2+}–Mg^{2+})-ATPase it is replaced by Asn (Green, 1989). Although it is therefore unlikely to be an essential residue, modification does result in a 59% loss of ATPase activity (Figure 1). The fluorescence of the ATPase modified at Lys-515 with fluorescein isothiocyanate has been shown to be sensitive to a variety of conformational changes on the ATPase, including those resulting from binding of Ca^{2+} and phosphorylation by ATP or P_i (Pick, 1981b). Fluorescence of the ATPase labeled with BrF, however, appears to be insensitive to Ca^{2+} binding or to phosphorylation, with any changes in intensity being less than 2% (data not shown); maximal levels of phosphorylation of the ATPase observed with [γ-^{32}P]ATP were unchanged by labeling with BrF (data not shown). Antipeptide antibody binding studies have defined much of the phosphorylation and nucleotide binding domains as being surface exposed, and it has been shown that antipeptide antibodies raised to peptides corresponding to residues 422–432 and 454–466 bind to the native ATPase (Mata et al., 1993), indicating that the region of the ATPase around Glu-439 is unlikely to be deeply buried. Figure 8 compares KI quenching of the fluorescence of free BrF with that for BrF-labeled ATPase. Johnson and Yguerabide (1985) have suggested that quenching constants for a bound fluorophore will be less than for the same fluorophore free in solution because the translational and rotational diffusion coefficients of the bound fluorophore will be smaller than those of the free fluorophore; they estimated that this would result in a reduction in Stern–Volmer quenching constant by a factor of 2. The data in Figure 8 fit to quenching constants of 7.4 and 2.7 M^{-1} for free and bound BrF, respectively, suggesting that the fluorescein bound at Glu-439 must be relatively accessible to the aqueous medium. This is also consistent with measurements of the pK value for the bound fluorophore, for which a value of 6.98 is obtained, very close to that obtained for the free BrF (6.83) (Figure 7).

Glu-439 can be located in the three-dimensional structure of the ATPase by measuring the efficiency of energy transfer to other labeled residues on the ATPase or to labeled phospholipids. It has been shown that at molar ratios of probe to protein of less than 1:1, EITC labels the ATPase at Lys-515 (Papp et al., 1987). The ATPase has been doubly labeled with BrF and EITC at various molar ratios of EITC to ATPase, and the efficiencies of energy transfer have been calculated (Figure 9, Table II). These have then been scaled to give an efficiency of transfer expected for 1:1 labeling with EITC; the observation that the same value is estimated extrapolating from three different EITC to ATPase labeling ratios is consistent with labeling of a single site on the ATPase by EITC under these conditions. From the efficiency of transfer, the distance between Glu-439 and Lys-515 is calculated to be 41–43 Å. Addition of the detergent $C_{12}E_8$ at concentrations (2.5 mg/mL) sufficient to solubilize the ATPase in monomeric form (Andersen et al., 1985) had no effect on the efficiency of energy transfer (data not shown), showing that the transfer was intramolecular. Measurements of energy transfer between coumarin at Cys-344 and fluorescein at Glu-439 give a distance between these residues of 40 Å (Table III). Finally, the height of the fluorescein label above the bilayer surface can be determined by measuring the efficiency of energy transfer to FITC-PE (Figure 10). As shown in Figure 10, the data fit well to the three theoretical approaches utilized. The methods of Koppel et al. (1979) and of Dewey and Hammes (1980) consider the donor and acceptor fluorophores as being located in two planes a distance h apart; the two approaches give distances of 68 and 70 Å, respectively (Figure 10). The approach of Gutierrez-Merino (Gutierrez-Merino, 1981; Gutierrez-Merino et al., 1987) characterizes the position of the label on the ATPase in terms of its height h above the membrane surface and the distance d between the site and the circumference of the protein. It has been shown elsewhere that when the protein donor is located far from the membrane surface, a wide range of values of h and d give equally good fits to the data as long as the values of d and h are chosen to keep the distance between the protein donor and the annular shells of phospholipids surrounding the ATPase a constant. As shown in Figure 10, a good fit to the data is obtained with d = 5 Å and h = 72 Å. None of these measured distances change on binding Ca^{2+} or vanadate. Similarly, it has been shown that the heights of Lys-515 and Cys-344 above the bilayer surface are unaltered by binding of Ca^{2+} or vanadate and that the separation between Cys-344 and Lys-515 is unaltered (Gutierrez-Merino et al., 1987; Mata et al., 1993). It has been suggested that phosphorylation of the ATPase involves a relative movement of the phosphorylation and nucleotide binding domains (Stahl & Jencks, 1987). The lack of relative movement of Cys-344 and Glu-439 in the phosphorylation domain and Lys-515 in the nucleotide binding domain suggests that any differences in the positions of these domains in the unliganded, Ca^{2+}-bound, or vanadate-bound forms is very small, despite the observed differences, for example, in susceptibility to proteolysis in these different states (le Maire et al., 1990).

Figure 11 shows distances measured by fluorescence energy transfer superimposed on the structure of the ATPase deduced by Stokes and Green (1990) from studies of negatively stained crystals of the ATPase. It has been argued elsewhere that the phospholipid–water interface as defined with RITC-PE will correspond to the level of the glycerol backbone of the phospholipid whereas electron microscopy of negatively stained membranes will define the surface as that of the phospholipid headgroup region (Munkonge et al., 1989; Mata et al., 1993). Since a phosphatidylcholine headgroup extends about 15 Å from the glycerol backbone in crystals of phospholipid (Pearson & Pascher, 1979), definitions of the membrane surface by fluorescence and electron microscopy will differ by about 15 Å (Figure 11). Location of Lys-515 80 Å above the glycerol backbone region (Gutierrez-Merino et al., 1987) would put Lys-515 on the top surface of the larger lobe of the ATPase, defining this lobe as the nucleotide binding domain, with Cys-344 and Glu-439 on the smaller lobe, representing the phosphorylation domain (Mata et al., 1993).

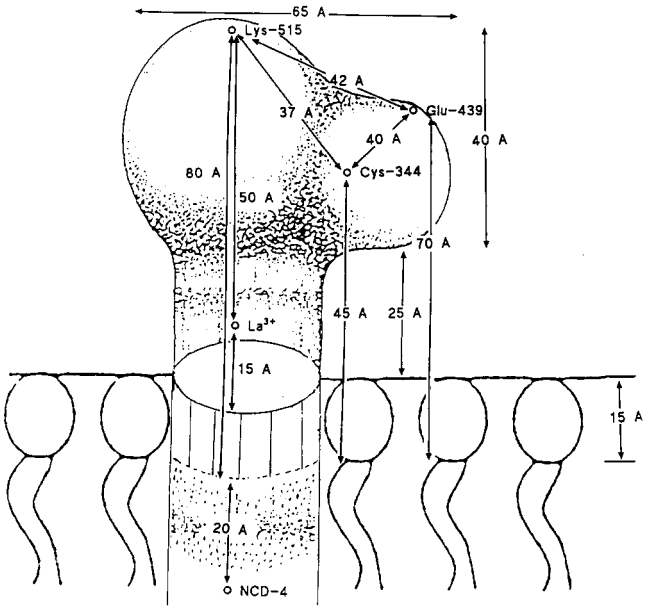

FIGURE 11: Location of residues on the ATPase as defined by fluorescence energy transfer. Positions of Lys-515, Cys-344, and Glu-439 are given on the structure deduced by Stokes and Green (1990) from studies of negatively stained crystals of the ATPase. Also shown are the locations of sites labeled by *N*-cyclohexyl-*N'*-(dimethylamino-1-naphthyl)carbodiimide (NCD-4), believed to be associated with the binding of Ca^{2+}, and a possible location for site(s) binding lanthanides (Mata et al., 1993).

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