Reactivity of Lysyl Residues on the (Ca^{2+}–Mg^{2+})-ATPase to 7-Amino-4-methylcoumarin-3-acetic Acid Succinimidyl Ester†

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ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum was labeled with the succinimidyl ester of 7-amino-4-methylcoumarin-3-acetic (AMCA). Although a large number of residues were labeled, it was found that Lys-492 was labeled preferentially at pH values between 6 and 8, consistent with an unusual environment for this residue. Labeling was reduced in the presence of ATP, suggesting that Lys-492 is in or near the ATP binding site of the ATPase. Other identified residues labeled by AMCA were Lys-35, Lys-135, Lys-218, Lys-371, and Lys-605. It is suggested that these represent surface-exposed lysyl residues. Lys-515, labeled by fluorescein isothiocyanate (FITC), was not labeled by AMCA. Labeling with AMCA at pH 6.0 has no effect on ATPase activity, suggesting that Lys-492 is not essential for activity. The fluorescence of AMCA-labeled ATPase did not change on addition of either ATP in the presence of Ca^{2+} or P_i in the absence of Ca^{2+}, suggesting that Lys-492 was not affected by any major conformational changes on the ATPase. The efficiency of fluorescence energy transfer between AMCA and FITC labels on the ATPase was unaffected by binding Ca^{2+} or vanadate, arguing against any large-scale movement of the cytoplasmic domains of the ATPase.

The (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum is one of a class of ion-transport ATPases which link the hydrolysis of ATP to the unidirectional movement of ions across biological membranes. An understanding of the mechanism of the ATPases will require information about their structure and about the relative locations of the sites for ATP hydrolysis and ion transport, as well as information about the conformational changes that underlie transport. One approach is to use reagents specific for particular functional groups on the ATPase to demonstrate which groups are essential for activity; if the reagent is fluorescent, then it may also be possible to use the fluorescence properties of the modified residues to detect conformational changes on the protein.

Chemical labeling has been used to identify a number of residues which may be part of the ATP binding site of the ATPase. Labeling of Lys-515 with fluorescein isothiocyanate (FITC)[1] is competitive with binding of ATP, and labeling leads to loss of ATPase activity although hydrolysis of acetyl phosphate is still possible, suggesting that Lys-515 is part of the nucleotide binding region (Mitchinson et al., 1982; Pick & Karlish, 1982). Adenosinetriphosphopyridoxal labels Lys-684 in the presence of Ca^{2+} and both Lys-684 and Lys-492 in the absence of Ca^{2+} (Yamamoto et al., 1988, 1989), consistent both with a change in the relative positions of Lys-684 and Lys-492 on binding Ca^{2+} and with a location for these two residues in the ATP binding site, possibly interacting with the phosphate groups of ATP (Yamamoto et al., 1989). Lys-492 has also been shown to be labeled by 2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azido-ATP (McIntosh et al., 1992), and Lys-492 and Arg-678 have been reported to be cross-linked by glutaraldehyde (McIntosh & Ross, 1992), each in an ATP-protectable manner, again consistent with Lys-492 being part of the ATP binding site.

Yamamoto and Tonomura (1976) modified lysyl residues in the (Ca^{2+}–Mg^{2+})-ATPase with 2,4,6-trinitrobenzenesulfonate and reported that one residue was modified in the presence of ATP, but that four residues were modified in the presence of Ca^{2+}; these residues have not been located in the sequence of the ATPase. Given the large number of lysyl residues in the (Ca^{2+}–Mg^{2+})-ATPase, the experiments of Yamamoto and Tonomura (1976) would suggest the presence of a small number of lysyl residues of particularly high reactivity. Furthermore, the pH dependence of inactivation of Lys-492 by (2,4,6-trinitrophenyl)-8-azido-ATP has been found to be consistent with a pK of 7.5 for this lysine, about 3 orders of magnitude lower than that for lysine in solution, suggesting an unusual environment in the ATPase (Seebregts & McIntosh, 1989). Here we report a study of labeling with succinimidyl esters which modify lysyl residues under mild conditions (Anderson et al., 1964). 7-Amino-4-methylcoumarin-3-acetic acid succinimidyl ester (AMCA) (Khalfan et al., 1986) has the advantage for these studies of introducing a fluorescent group into the ATPase, potentially of use in studying conformational changes on the ATPase.

MATERIALS AND METHODS

7-Amino-4-methylcoumarin-3-acetic acid succinimidyl ester (AMCA) and fluorescein isothiocyanate (FITC) were obtained from Molecular Probes and Aldrich, respectively. 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 as 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

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[1] Abbreviations: AMCA, 7-amino-4-methylcoumarin-3-acetic acid succinimidyl ester; FITC, fluorescein isothiocyanate.

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initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents.

Labeling with AMCA. Purified ATPase was suspended to 10–12 mg/mL in buffer at pH 6.0 (40 mM Mes/Tris) or pH 7.0 or 8.0 (40 mM Tris/Mes) and incubated in the dark at 15 °C for 2 h with AMCA at AMCA:ATPase molar ratios of 5:1 (pH 6.0), 10:1 (pH 7.0), or 15:1 (pH 8.0); AMCA was added from a 30 mM stock solution in dimethylformamide. Unbound AMCA was separated from the labeled ATPase by centrifugation through Sephadex G-50 columns preequilibrated with the above buffers (Munkonge et al., 1989). Concentrations of ATPase were estimated from the absorbance at 280 nm in 1% SDS and NaOH (0.1 M) using the extinction coefficient given by Hardwicke and Green (1974), and the amount of AMCA covalently bound to the ATPase was estimated from the absorbance at 355 nm, using an extinction coefficient of 15 000 M^{-1} cm^{-1}. Where appropriate, the measured absorbance at 280 nm was corrected for the small absorbances of the fluorescence probes at this wavelength before estimation of the concentration of the ATPase.

ATPase was labeled with FITC by incubation of FITC and ATPase at a molar ratio of 2:1 in 40 mM Tris/Mes, pH 8.0, for 30 min at room temperature, followed by separation of unreacted FITC by centrifugation through Sephadex G-50. Double-labeled ATPase was prepared either by first labeling with AMCA followed by removal of unreacted AMCA and reaction with FITC, using the protocols described above, or by first labeling with FITC followed by removal of unreacted FITC and reaction with AMCA. Concentrations of FITC were determined following solubilization of samples in 1% SDS and NaOH (0.1 M), using an extinction coefficient of ϵ_{500} = 80 000 M^{-1} cm^{-1} (Gutierrez Merino et al., 1987).

Proteolysis of the ATPase. Labeled ATPase (5 mg/mL) was incubated with thermolysin (Sigma, protease type X) at room temperature in 100 mM NH_4HCO_3, pH 7.7, in a total volume of 500 μL at a ratio of thermolysin to ATPase of 50:1 (mg/mg). The digestion was terminated after 3 h by centrifugation at 14000*g* for 2.5 min. The supernatant was separated, and the pellet was washed with 100 μL of acetonitrile 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 the supernatant were recorded in 40 mM Hepes/KOH, pH 7.0, using a Perkin Elmer LS-3B fluorometer with excitation and emission wavelengths of 355 and 442 nm, respectively. An estimated 80–85% of the AMCA label was present in the supernatant.

Separation of peptides was performed using an Applied Biosystems gradient HPLC system 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 two gradient systems. The first was made from 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. The second was made from 10 mM aqueous potassium phosphate (adjusted to pH 7.7 with trifluoroacetic acid) and a mixture of this potassium phosphate solution with acetonitrile in a ratio of 1:9 (v/v), respectively.

Peptide Sequencing. Amino acid sequences of AMCA–peptides were determined using a pulsed-liquid sequencer (Applied Biosystems, Model 477A). The PTH derivative released at each cycle of the degradation was split into two samples, one of which was applied to an on-line PTH analyzer (Applied Biosystems, Model 120A), and the other was analyzed for fluorescence using a Perkin Elmer LS-3B fluorometer.

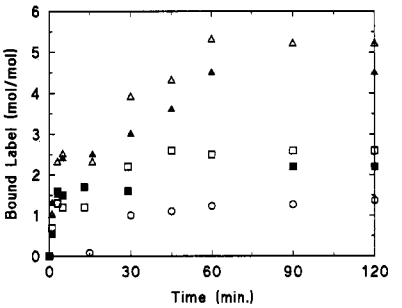

FIGURE 1: Modification of the ATPase by AMCA. The (Ca^{2+}–Mg^{2+})-ATPase (○, □, △) or FITC-labeled ATPase (■, ▲) (174 μM) was incubated with AMCA (2.6 mM) at pH 6.0 (○), 7.0 (□, ■), or 8.0 (△, ▲), 15 °C, for the given time, and unreacted AMCA was separated from labeled ATPase on Sephadex G-50. The ratio of bound label to ATPase was estimated by the absorbance.

Table I: Labeling of the (Ca^{2+}–Mg^{2+})-ATPase by AMCA[a]

pH	conditions	label:ATPase ratio	% activity[b]
6	–MgATP	1.28	92
	+MgATP	0.9	100
	+AMCA[c]	2.17	
7	–MgATP	2.73	31
	+MgATP	2.44	38
8	–MgATP	6.3	17
	+MgATP	5.2	29

[a] The ATPase was incubated with AMCA at molar ratios of dye to ATPase of 10:1 (pH 6.0 and 7.0) or 15:1 (pH 8.0) in 40 mM Hepes, pH 6.0, or 40 mM Tris/Mes, pH 7.0 or 8.0, containing 1 mM EGTA, 5 mM $MgSO_4$, and, where indicated, 5 mM MgATP, for 2 h at 15 °C. [b] Activities measured at pH 7.2 relative to that of the unlabeled ATPase treated under the same conditions. For pH 7.0 and 8.0, incubation of the ATPase under the conditions used for labeling in the absence of AMCA had no effect on activity, whereas at pH 6.0 activity decreased after 2 h to 77% of the original activity. The original ATPase activity was 3.5 IU/mg of protein measured at pH 7.2, 25 °C. [c] An additional aliquot of AMCA at a 5:1 molar ratio of label to ATPase was added after 1 h.

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/Mes 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.

RESULTS

Modification of (Ca^{2+}–Mg^{2+})-ATPase with AMCA. When the purified (Ca^{2+}–Mg^{2+})-ATPase was incubated with excess AMCA, coumarin label was incorporated, with the level of incorporation increasing with increasing pH (Figure 1, Table I). The incorporation of label reached a constant level at long times, but this is not due to saturation of sites on the ATPase, but rather to hydrolysis of the AMCA label in solution, a reaction in competition with the labeling reaction. If AMCA is incubated in buffer in the absence of ATPase, decomposition occurs as shown by the appearance of new spots on thin-layer chromatography and new peaks in HPLC traces; for example, after 1 h at pH 8.0, decomposition of AMCA is complete (data not shown). This is also confirmed by the observation shown in Table I that if additional AMCA is added to the ATPase after an initial incubation with AMCA at pH 6.0, then further labeling of the ATPase is observed. Comparable levels of labeling were observed either in the presence of EGTA or with 1 mM Ca^{2+} (data not shown).

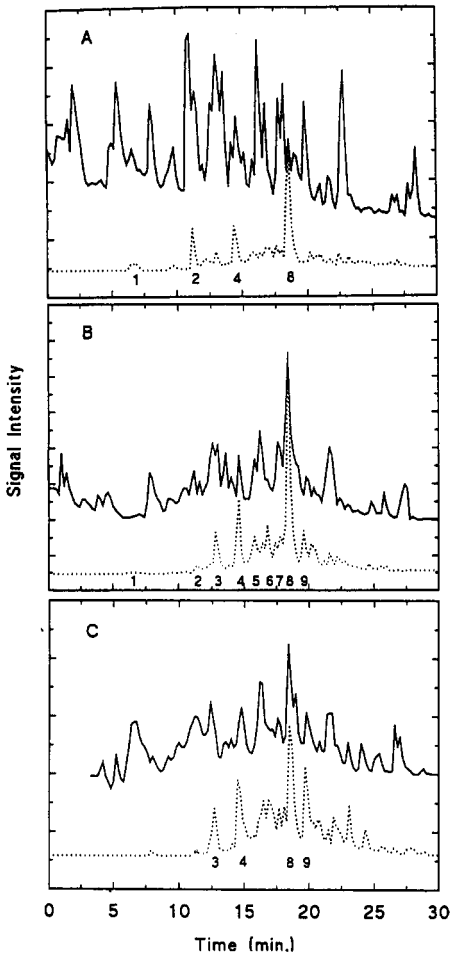

FIGURE 2: Analysis of peptide fragments by reversed-phase HPLC. Soluble peptides obtained by thermolysin cleavage of the ATPase labeled with AMCA at pH 6.0 (A) or at pH 8.0 in the absence (B) or presence of ATP (C) were fractionated on a C_{18} column at a flow rate of 1 mL/min. The peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, from 10 to 40% acetonitrile. The top trace shows the absorbance measured at 235 nm, and the lower trace shows the fluorescence, measured with excitation and emission wavelengths of 350 and 425 nm, respectively.

The effect of AMCA on the activity of the ATPase is shown in Table I. Incubation of the ATPase under the conditions of labeling at pH 6.0 but in the absence of AMCA followed by passage through a column of Sephadex G-50 leads to a decrease in ATPase activity of 23%. The same decrease in activity is observed on incubation with AMCA at pH 6.0, indicating that labeling at pH 6.0 has no effect on ATPase activity. A similar decrease in ATPase activity on incubation at acid pH values has been reported by Berman et al. (1977). In contrast, incubation under the conditions of labeling at pH 7.0 or 8.0 in the absence of AMCA has no effect on activity, whereas incubation with AMCA at pH 7.0 or 8.0 results in a large decrease in activity (Table I).

Identification of Residues Labeled by AMCA. The purified ATPase was incubated with excess AMCA at pH 6.0, 7.0, or 8.0 for 2 h and excess AMCA removed by passage through Sephadex G-50. The ATPase was then partially digested with thermolysin, and soluble peptides were separated from membranous fragments by centrifugation. As estimated by fluorescence measurements, more than 85% of the initial bound coumarin 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 2A, for the ATPase labeled with AMCA at pH 6.0, fluorescence is detected in four major peaks and in a large number of minor peaks; the peaks are numbered in Figure 2 for ease of comparison between labeling experiments at different pH values. Unreacted AMCA was shown in separate experiments to give two peaks eluting at 7 and 12 min, corresponding to peaks 1 and 2 in Figure 2A; 1-mL fractions were collected from the HPLC column and the relative fluorescences of the major peaks determined. Peaks 4 and 8 accounted for 10 and 31% of the bound fluorescence, respectively. As shown in Figure 2B, a very similar pattern of fluorescent peaks is observed following proteolysis of the ATPase labeled with AMCA at pH 8.0, but now the minor peaks are observed more clearly. Again, 1-mL fractions were collected, and the relative fluorescences of the major peaks were determined, with the results given in Table II.

Table II: Relative Fluorescences of the Major Peaks Obtained from HPLC Separation of Peptides Obtained from Proteolysis of the ATPase Labeled with AMCA at pH 8.0 in the Presence or Absence of ATP

	−ATP		+ATP[a]	
peak	% fluorescence[b]	label ratio[c] (mol/mol)	% fluorescence[b]	label ratio[d] (mol/mol)
1	0.9	0.06	1.0	0.05
2	1.7	0.11	2.0	0.10
3	4.8	0.30	6.0	0.31
4	9.1	0.57	10.6	0.55
5	5.2	0.33	11.6	0.60
6	9.0	0.57		
7	7.8	0.49	9.2	0.48
8	18.0	1.13	12.4	0.64
9	7.3	0.46	10.9	0.57

[a] Labeling performed in the presence of 5 mM ATP. [b] Percent of total fluorescence of the fractions recovered from the HPLC column; this amounted to ca. 80% of the initial fluorescence of the peptide mixture applied to the HPLC column. [c] Calculated on the basis of the initial molar ratio of bound coumarin to ATPase of 6.3:1. [d] Calculated on the basis of the initial molar ratio of bound coumarin to ATPase of 5.2:1.

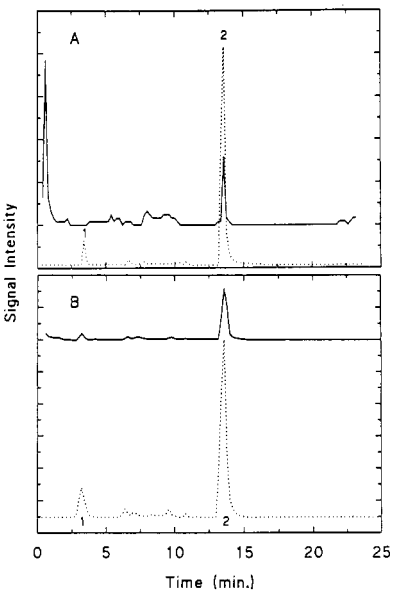

FIGURE 3: Purification of peptide fragments by reversed-phase HPLC. Peak 8, obtained as shown in Figure 2A,B, respectively, from the ATPase labeled with AMCA at pH 6.0 (A) and 8.0 (B), was further fractionated on a C_{18} column at a flow rate of 1 mL/min, with a linear gradient of acetonitrile in 10 mM potassium phosphate (pH 7.5), from 15 to 40% acetonitrile. The top and bottom traces show the absorbance and fluorescence measured as described in the legend to Figure 2.

The major peaks were further purified by reverse-phase HPLC with an acetonitrile gradient in 10 mM potassium phosphate (pH 7.5). As shown in Figure 3A, the major peak obtained from the ATPase labeled at pH 6.0 (peak 8 in Figure 2A) gave one major peak accounting for 86% of the

Table III: Sequences and Purified Labeled Peptides

pH	peak number: first column	peak number: second column	sequence[a]	labeled Lys[b]
6	8	2	FSRDRXS	492
7	3	1	ADRXS	135
		2	ADRXSVQRT	135
	4	3	RXSVQ	135
	8	2	FSRDRXS	492
8	3	1	ADRXS	135
		3	AGXA	218
	4	3	RXSVQ	135
	5	3	SRDRXS	492
	8	1	LEXY	35
		2	FSRDRXS	492
	9	1	LDPPRXE	605
		2	FIIDXV	371

[a] X represents the position of a PTH-amino acid derivative which elutes from the reverse-phase column in a position similar to PTH-tryptophan, and exhibiting coumarin fluorescence. [b] Numbered by comparison to the published sequence of the ATPase (Brandl et al., 1986).

fluorescence and one minor peak. The major peak was collected and sequenced with the result shown in Table III. Peak 4 shown in Figure 2A was found to contain several peptides not separating well on the second column, and sequences that were obtained suggested different cuts of the peptide sequences from peak 8 (data not shown).

The major peak obtained from the ATPase labeled at pH 8.0 (peak 8 in Figure 2B) also gave one major peak accounting for 76% of the fluorescence when further purified (Figure 3B). The sequence of this peptide is the same as that of the major peak obtained from the ATPase labeled at pH 6.0 (Table III). Peaks 3, 4, 5, and 9 (Figure 2B) were also further purified with the results shown in Figure 4. Peak 3 was found to contain four major fluorescent components of which peaks 1 and 3 gave the sequences in Table III; peak 2 gave no clear sequence, and peak 4 contained a number of overlapping components so that sequencing was not attempted. Peak 4 contained a single major component with the sequence given in Table III. Peak 5 contained at least five fluorescent components; the major component (peak 3) was sequenced with the result shown in Table III. Peak 9 contained two major components whose sequences are again given in Table III. The minor peaks 6 and 7 in Figure 2B were found to contain a number of components, for which sequencing was not attempted.

Proteolysis of the ATPase labeled with AMCA at pH 7.0 gave a pattern of labeling very similar to that shown in Figure 2B for labeling at pH 8.0 (data not shown). The major peaks corresponding to peaks 3, 4, and 8 in Figure 2B were further purified as described above. Peak 3 was again found to contain four major components (as shown in Figure 4 for labeling at pH 8.0). In this case, both of the major peaks 1 and 2 gave sequences, given in Table III. As for the ATPase labeled at pH 8.0, peaks 4 and 8 contained single major components, with sequences given in Table III.

Effects of Labeling with FITC or Labeling in the Presence of ATP. As shown in Figure 1, if the ATPase is first labeled with FITC and then labeled with AMCA at pH 7.0 or 8.0, a lower level of incorporation of AMCA is observed than in the absence of prelabeling with FITC. As shown in Table I, addition of ATP (5 mM) to the labeling medium also results in reduced incorporation of AMCA. Figure 2C shows the pattern of labeled peptides obtained after proteolysis of the ATPase labeled with AMCA at pH 8.0 in the presence of ATP, and Table II compares the relative fluorescence intensities in the major peaks with those obtained in the absence of ATP. The major effect of ATP is to reduce the labeling of peak 8. In the experiment shown in Figure 2C, peaks 5 and 6 were not obtained separately when 1-mL fractions were collected for fluorescence analysis, so only a combined figure can be given for these two bands; the data suggest that labeling of this combined fraction might be reduced (Table II).

Although as shown in Figure 1 prelabeling the ATPase with FITC reduces labeling with AMCA at pH 8.0, labeling with AMCA does not prevent labeling with FITC. This is illustrated in the absorption spectra shown in Figure 5. When the ATPase was first labeled with excess AMCA at pH 8.0 to give a bound coumarin:ATPase molar ratio of 6.1:1 (Figure 5, dashed line) and then labeled with FITC, FITC was incorporated to a FITC:ATPase ratio of 0.85:1 (Figure 5, solid line).

Fluorescence Properties of AMCA-Labeled ATPase. The coumarin fluorophore on AMCA-labeled ATPase exhibits fluorescence centered at 440 nm when excited at 320 nm. Spectra recorded for the ATPase labeled to different molar ratios of AMCA to ATPase showed no significant differences, apart from the expected changes in fluorescence intensity. Figure 6 shows the fluorescence response of AMCA-labeled ATPase to the addition of Mg^{2+} and P_i at pH 6.0 in the absence of Ca^{2+} and to the addition of ATP in the presence of Mg^{2+} and Ca^{2+}. Whereas the fluorescence intensity of the ATPase labeled at pH 6.0 increases on addition of 20 mM Mg^{2+} (by 2%, when corrected for dilution), the fluorescence intensity of the ATPase labeled at pH 7.0 decreases by 1.3%. The decrease in signal intensity on subsequent addition of P_i is equal to that expected from the dilution of the sample. Addition of ATP in the presence of Ca^{2+} and Mg^{2+} results in a change in fluorescence intensity of less than 1%, as shown in Figure 6. Effects of addition of Ca^{2+} on fluorescence intensity are also 1% or less (data not shown).

For the ATPase labeled first with AMCA and then with FITC, fluorescence energy transfer was observed between coumarin and fluorescein labels as shown by a decrease in fluorescence intensity for the coumarin label in the doubly-labeled system at any given molar ratio of coumarin to ATPase (Figure 7). As shown in Figure 7, the proportional reduction in coumarin fluorescence intensity on labeling with FITC is the same for the ATPase labeled for 2 h with AMCA at pH 6 to give a molar ratio of bound coumarin to ATPase of 1.3:1 or for the ATPase labeled at pH 8.0 to give a molar ratio of bound coumarin to ATPase of 5:1. Efficiences of transfer, calculated as $(F_0 - F)/F_0$, where F_0 and F are the fluorescence intensities for coumarin in the singly- and doubly-labeled species, respectively, were 0.65, 0.65, and 0.67 for labeling at pH 6, 7, and 8, respectively. Addition of Ca^{2+} or vanadate had no measurable effect on the efficiencies of transfer (data not shown).

DISCUSSION

The (Ca^{2+}–Mg^{2+})-ATPase appears in electron micrographs as a bilobed structure connected to the membrane by a narrow stalk (Dux et al., 1985; Stokes & Green, 1990). Molecular modeling of the ATPase based on its sequence has suggested a structure containing three main cytoplasmic domains: a nucleotide binding domain where ATP binds, a phosphorylation domain containing the aspartyl residue (Asp-351) on the ATPase that is phosphorylated by ATP or P_i, and a β-strand or transduction domain which serves to link phosphorylation of the ATPase to transport of Ca^{2+}. A small hinge or central domain links the nucleotide binding domain to the stalk region (MacLennan et al., 1985; Brandl et al., 1986).

Labeling of Lys-515 with FITC is competitive with binding of ATP and prevents phosphorylation of the ATPase by ATP,

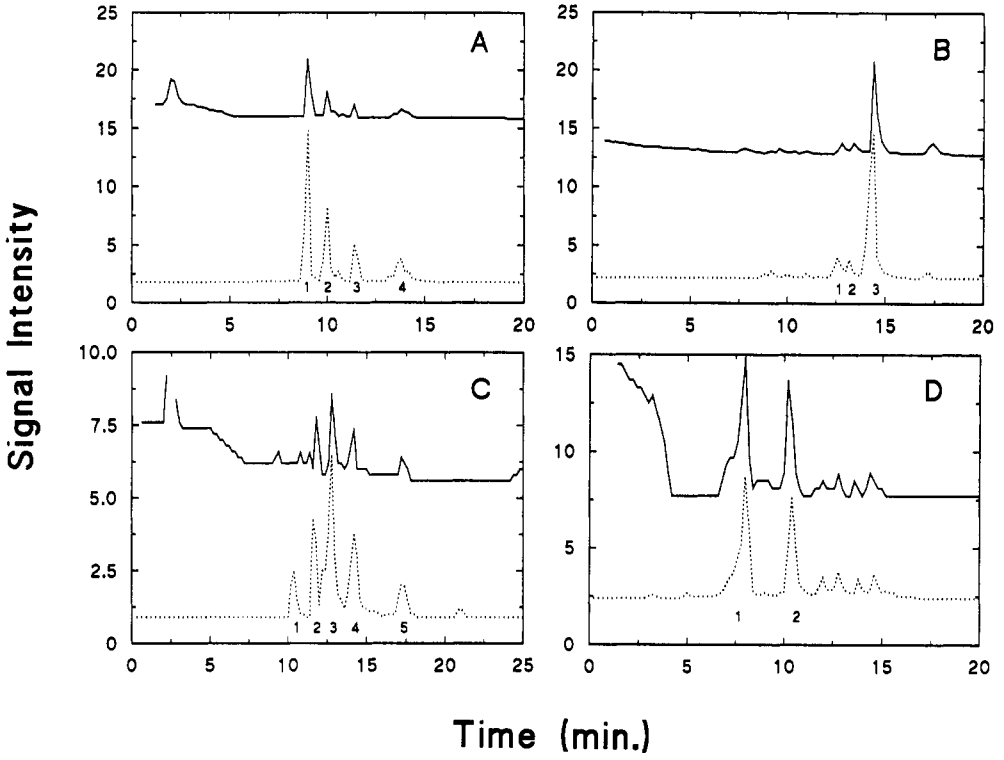

FIGURE 4: Purification of peptide fragments by reversed-phase HPLC. Peaks 3 (A), 4 (B), 5 (C), and 9 (D), obtained as shown in Figure 2B from the ATPase labeled with AMCA at pH 8.0, were further fractionated on a C_{18} column at a flow rate of 1 mL/min, with a linear gradient of acetonitrile in 10 mM potassium phosphate (pH 7.5), from 10 to 30% (peaks 3,4), from 10 to 35% (peak 5), or from 15 to 35% acetonitrile (peak 9). The top and bottom traces show the absorbance and fluorescence measured as described in the legend to Figure 2.

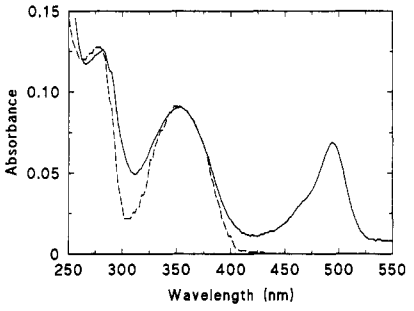

FIGURE 5: Absorbance spectra of the ATPase labeled with FITC following labeling with AMCA. The ATPase was first labeled with excess AMCA at pH 8.0 to give a bound coumarin:ATPase molar ratio of 6.1:1 (dashed line) and then labeled with FITC to a bound FITC:ATPase molar ratio of 0.85:1 (solid line). Spectra were recorded in 1% SDS and NaOH (0.1 M). Excess unbound label was removed by passage through Sephadex G-50, and the concentration of ATPase was 1.0 μM.

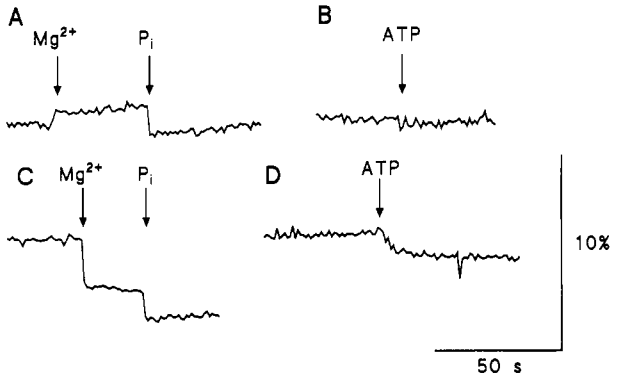

FIGURE 6: Changes in the fluorescence intensity of the ATPase labeled with AMCA at pH 6.0 (A, B) or pH 7.0 (C, D). In (A) and (C), the labeled ATPase was incubated in 40 mM Mes/Tris, pH 6.0, and 1 mM EGTA (3 mL), and at the arrows, stock solutions of Mg^{2+} (30 μL) or P_i (60 μL) were added to give final concentrations of 20 mM Mg^{2+} and 20 mM P_i. In (B) and (D), the labeled ATPase was incubated in 40 mM Mes/Tris, pH 7.0, 5 mM Mg^{2+}, and 10 μM free Ca^{2+} (3 mL), and at the arrow, a stock solution of ATP (6 μL) was added to give an ATP concentration of 20 μM.

suggesting that Lys-515 is part of the nucleotide binding site (Mitchinson et al., 1982; Pick & Karlish, 1982). Although this is a conserved residue in the ATPases (Green, 1989), it has been shown by site-directed mutagenesis not to be essential for activity (Maruyama et al., 1989). We have determined the distance between Lys-515 and the lipid–water interface as about 80 Å, putting Lys-515 on the top surface of the ATPase (Gutierrez-Merino et al., 1987). Immunological studies have demonstrated considerable surface exposure in the postulated nucleotide binding domain, also consistent with a location for this domain on the top surface of the ATPase (Mata et al., 1992a).

Adenosinetriphosphopyridoxal labels Lys-684 in the (Ca^{2+}–Mg^{2+})-ATPase in the presence of Ca^{2+} and both Lys-684 and Lys-492 in the absence of Ca^{2+} (Yamamoto et al., 1988, 1989). It has therefore been suggested that the relative positions of Lys-684 and Lys-492 are affected by a Ca^{2+}-dependent conformational change and that both are located in the ATP binding site, possibly interacting with the phosphate groups of ATP (Yamamoto et al., 1989). Lys-492 has been shown to be labeled by 2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azido-ATP (McIntosh et al., 1992) in an ATP-protectable manner. It is a conserved residue, and the equivalent residues in lamb kidney (Na^+–K^+)-ATPase (Lys-480) and pig gastric (H^+–K^+)-ATPase (Lys 497) are labeled by pyridoxal phosphate (Hinz & Kirley, 1990; Tamura et al., 1989). In dog kidney (Na^+–K^+)-ATPase, it has been shown that FITC can react with Lys-501, Lys-480, or Lys-766, but with only one per ATPase molecule; it has therefore been suggested that these residues [equivalent to Lys-515, Lys-492, and Lys-758 in the (Ca^{2+}–Mg^{2+})-ATPase] are clustered around the binding site for fluorescein (Xu, 1989). McIntosh and Ross (1992) have reported that glutaraldehyde cross-links Lys-492 and Arg-

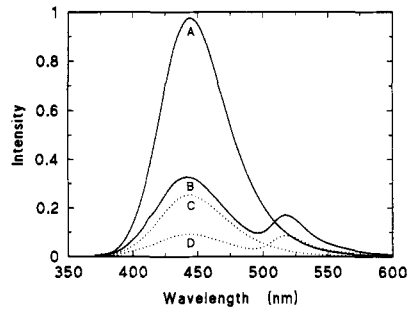

FIGURE 7: Fluorescence emission spectra for the ATPase doubly-labeled with AMCA and FITC. Spectra are shown for the ATPase labeled at pH 8.0 (A) and 6.0 (B) with AMCA to give bound coumarin:ATPase molar ratios of 5:1 and 1.3:1, respectively. Following labeling with AMCA at pH 8.0 (C) and 6.0 (D), samples were then labeled with FITC, to give a bound fluorescein:ATPase molar ratio of 0.9:1. The concentration of ATPase for all spectra was 0.03 μM, and spectra were run in 40 mM Hepes/KOH, pH 7.2, 100 mM NaCl, and 1 mM EGTA.

678 in the (Ca^{2+}–Mg^{2+})-ATPase, in an ATP-protectable manner, again consistent with Lys-492 being part of the ATP binding site. Ohta et al. (1986) have reported that 5′-[(*p*-(fluorosulfonyl)benzoyl]adenosine labels a lysine in the (Na^+–K^+)-ATPase equivalent to Lys-712 in the (Ca^{2+}–Mg^{2+})-ATPase, suggesting this Lys is also in the ATP binding site; mutation of this residue in the (Ca^{2+}–Mg^{2+})-ATPase has no effect on activity, so that its role cannot be essential (Maruyama et al., 1989). Asp-703 and Asp-707 in the (Na^+–K^+)-ATPase, conserved residues equivalent to Asp-702 and Asp-706 in the (Ca^{2+}–Mg^{2+})-ATPase, are labeled by adenosine 5′-*N*-[4-[*N*-(2-chloroethyl)-*N*-methylamino]benzyl]-γ-amidotriphosphate, so that these residues are also presumably part of the ATP binding site (Dzhandzhugazyan et al., 1988).

Yamamoto and Tonomura (1976) suggested that a limited number of lysyl residues in the (Ca^{2+}–Mg^{2+})-ATPase were modified by 2,4,6-trinitrobenzenesulfonate, but these residues have not been located. We have chosen to study modification of lysyl residues with the succinimidyl ester of 7-amino-4-melthylcoumarin-3-acetic (AMCA) both because of the mild conditions required for labeling with succinimidyl esters (Anderson et al., 1964) and because of the possible use of the introduced coumarin as a fluorescent reporter group. AMCA modifies more than one lysyl residue on the ATPase, reaction with the ATPase being in competition with decomposition of the AMCA (Figure 1, Table I). At pH 6.0, labeling at an AMCA:ATPase molar ratio of 10:1 results in a bound label:ATPase ratio of 1.3:1 (Table I). Sequencing demonstrated that the major residue labeled was Lys-492, this accounting for about 30% of the label incorporated. At pH 8.0, the most fluorescent peak obtained on the first HPLC separation of thermolysin-generated fragments of the ATPase (peak 8 in Figure 2B) was found to contain two major labeled components, of which the major component, representing 76% of the fluorescence, was the same peptide (FSRDRKS) as obtained after labeling at pH 6.0, containing Lys-492. This same labeled lysyl residue was observed in another peptide (SRDRKS) purified from peak 5 (Table III); this peptide contained 35% of the fluorescence of peak 5. On the basis of analysis of the fluorescence of the peptide fragments shown in Table III, the incorporation of AMCA into Lys-492 at pH 8.0 was 97% complete.

Labeling the ATPase with AMCA in the presence of ATP results in reduced incorporation of label (Table I), amounting at pH 8.0 to a reduction of 1.1 label per ATPase. As shown in Figure 2C and Table II, the major change is in the label content of peak 8 obtained in the first HPLC separation of proteolytic fragments, with some reduction also in peaks 5 plus 6, which were not resolved in this experiment. It is clear, therefore, that the presence of ATP reduces the labeling of Lys-492 by AMCA.

The preferential labeling of Lys-492 at all pH values suggests an unusual reactivity for this residue. This is consistent with the reported pH dependence of inactivation of Lys-492 by (2,4,6-trinitrophenyl)-8-azido-ATP which suggests a p*K* of 7.5 for this lysine, about 3 orders of magnitude lower than that for lysine in solution (Seebregts & McIntosh, 1989). The reduced labeling of this residue in the presence of ATP suggests that it is part of the ATP binding site on the ATPase, in agreement with labeling studies using a variety of ATP analogues (Yamamoto et al., 1988, 1989; McIntosh et al., 1992). Although labeling the ATPase at pH 6.0 with AMCA results in ca 40% labeling of this residue, it has no effect on ATPase activity (Table I). Mutation of the corresponding Lys (Lys-480) in the (Na^+–K^+)-ATPase has also been reported to have no effect on activity (Wang & Farley, 1992). Thus, despite its unusual properties, Lys-492 is unlikely to play an essential role in the function of the ATPase.

The second most labeled residue at pH 8.0 is Lys-135, contained in two purified peptides, ADRKS and RKSVQ, found in peaks 3 and 4; the labeling ratio for this residue is ca. 0.6:1. For the ATPase labeled with AMCA at pH 7.0, two labeled lysines were recognized in the recovered peptides: Lys-492 and Lys-135 (Table III), consistent with these being the two most reactive lysyl residues in the (Ca^{2+}–Mg^{2+})-ATPase. As shown by the data in Table II, the presence of ATP during labeling provides no protection for this residue, suggesting that it is not part of the ATP binding site. Lys-135 lies between the stalk region and the transduction domain in the model of MacLennan et al. (Dux et al., 1985; Stokes & Green, 1990). Little is known about this region of the ATPase, except that the T_2 tryptic cleavage site at Arg-198 is surface-exposed, as shown by the binding of antipeptide antibodies to the native ATPase (Tunwell et al., 1991).

As pointed out by a referee, the most highly labeled lysines (Lys-492 and Lys-135), as well as Lys-605, are all preceded by arginine, and are the only three RK sequences in the ATPase. Since lysines in the two KR and four KK sequences in the ATPase were not found to be labeled, this could suggest a conformational preference for the RK pair, bringing the charges on lysine and arginine close together, with a reduction in the p*K* for the lysine. Surface exposure is also likely to be a factor in the labeling of some lysyl residues by AMCA. Of those identified following labeling at pH 8.0, Lys-371 and Lys-605, with labeling ratios in the recovered, sequenced peptides of ca. 0.2, are in surface-exposed regions as defined by antibody binding experiments (Tunwell et al., 1991; Mata et al., 1992a), and Lys-35, also with a labeling ratio of ca. 0.2 in identified peptides, is probably surface-exposed, since the N-terminus has been shown to be exposed (Matthews et al., 1989). The remaining identified labeled lysyl residue, Lys-218, with a labeling ratio of ca. 0.2 in the identified peptides, is also likely to be surface-exposed since an antipeptide antibody raised to a peptide corresponding to residues 191–205 binds to the native ATPase (Tunwell et al., 1991) and since a tryptic cleavage site has been located at Arg-219 (Imamura & Kawakita, 1989). About 40% of the bound AMCA is located in the peptides that have been successfully sequenced (Tables II and III). Much of the remaining label is likely to be distributed among a large number of other lightly labeled lysyl residues.

On the basis of antibody binding experiments, we have suggested that fluorescein bound at Lys-515 in FITC-labeled

ATPase is buried in the native ATPase (Mata et al., 1989). Consistent with a preferential reaction of AMCA with surface-exposed residues, we find no evidence for labeling Lys-515 with AMCA (Table III). Although reduced labeling of the ATPase by AMCA is observed if the ATPase is first labeled with FITC (Figure 1), labeling first with AMCA does not prevent subsequent labeling by FITC (Figure 5). We conclude that AMCA does not react with Lys-515 and that the reduced labeling by AMCA observed for FITC-labeled ATPase is a result of steric hindrance by the relatively bulky fluorescein group.

In previous studies, we have shown that 4-(bromomethyl)-6,7-dimethoxycoumarin specifically labels the ATPase at Cys-344 and that the fluorescence of the labeled ATPase changes on binding Mg^{2+} and on phosphorylation with either P_i or ATP (Stefanova et al., 1992). For the ATPase labeled with AMCA at pH 6.0 or 7.0, small changes in fluorescence intensity are observed on binding Mg^{2+} (Figure 6), emphasizing the importance of Mg^{2+} in the functioning of the ATPase. However, no changes in fluorescence are seen on addition of either ATP in the presence of Ca^{2+} or P_i in the absence of Ca^{2+} (Figure 6). Since labeling the ATPase with AMCA at pH 6.0 does not inhibit ATPase activity (Table I), this suggests that the coumarin fluorescence of labeled Lys-492 is insensitive to phosphorylation, suggesting a location for Lys-492 away from Asp-351, the residue phosphorylated, and in a region unaffected by major conformational changes on phosphorylation of the ATPase. Similarly, no change in fluorescence intensity was observed for the labeled ATPase on binding Ca^{2+}, despite the change in reactivity of Lys-492 to adenosinetriphosphopyridoxal observed by Yamamoto et al. (1988, 1989).

Stahl and Jencks (1987) have suggested a conformational change on the ATPase following binding of ATP to the ATPase in the presence of Ca^{2+}, serving to relocate the nucleotide and phosphorylation domains on the ATPase, bringing the γ-phosphate of ATP close to Asp-351. We have measured distances between the lipid–water interface and Lys-515 and Cys-344 and between Lys-515 and Cys-344 in Ca^{2+}-free, Ca^{2+}-bound, and vanadate-bound forms by fluorescence energy transfer to estimate the extent of any structural change between major conformational states of the ATPase; we failed to detect any differences between any of these forms, suggesting that conformational changes are localized to small regions of the ATPase (Gutierrez-Merino et al., 1987; Mata et al., 1992b). For the ATPase doubly-labeled with AMCA and FITC, efficiencies of fluorescence energy transfer were also unaffected by addition of Ca^{2+} or vanadate (data not shown), again suggesting that conformational changes on the ATPase are restricted in nature. For the coumarin–fluorescein pair, the distance R_0 at which fluorescence energy transfer is 50% efficient has been calculated to be 37.5 Å (Mata et al., 1992b). The transfer efficiencies observed for the doubly-labeled ATPase would then correspond to an "average" coumarin–fluorescein separation of 34 Å on the ATPase.

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