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Molecular Properties of Calcium-Pumping ATPase from Human Erythrocytes†

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ABSTRACT: The Ca^{2+}-pumping ATPase from human erythrocyte membranes, purified by the method previously reported [Niggli, V., Penniston, J. T., & Carafoli, E. (1979) *J. Biol. Chem. 254*, 9955–9958], was freed of minor impurities by extensive washing while bound to the calmodulin–Sepharose column. The pure enzyme showed a single band of M_r 138 000, which contained no stainable carbohydrate. The enzyme retained calmodulin-stimulable ATPase activity; with appropriate assay conditions, an activity of 21.2 μmol/(mg·min) was obtained. Amino acid analysis showed that the ATPase had a larger proportion of polar amino acids than do other integral membrane proteins. Despite this, the ATPase showed a tendency to form dimers and higher aggregates even in the presence of sodium dodecyl sulfate and urea. The enzyme required Mg^{2+} but showed little activity unless a second ion was added. With regard to this second ion, the enzyme responded to alkaline earth metal ions in the order $Ca^{2+} > Sr^{2+} \gg Ba^{2+}$. It was highly specific for ATP and was stimulated by Na^+ or K^+; in all of these properties it resembled the enzyme in unfractionated membranes. Limited proteolysis using trypsin yielded, at short times, many fragments of various molecular weights; continued proteolysis resulted in two trypsin-resistant fragments of M_r 81 000 and 33 500. Analysis of the time course of proteolysis indicated that the ATPase existed in two or more conformations that had differing susceptibilities to proteolysis. It is suggested that these correspond to active and inactive conformers of the enzyme.

Several laboratories have established that Ca^{2+}–ATPase is responsible for active Ca^{2+} extrusion across the plasma membrane of human erythrocytes (Schatzmann, 1973; Schatzmann & Vincenzi, 1969; Roufogalis, 1979; Carafoli et al., 1980). Its enzymatic and physiological properties have been widely studied with red blood cell membranes, but only recently has it been purified nearly to homogeneity (Niggli et al., 1979). Because of the low concentration of this enzyme in the membrane, it has been difficult to free it of minor impurities; in the original preparation (Niggli et al., 1979) about 6% of the stain appeared in a position corresponding to band 3 (90 000 daltons).

In this paper we report a method for removing minor impurities from the enzyme. We also report chemical and catalytic properties of the purified enzyme, the absence of stainable carbohydrate in the enzyme, and the probable existence of two or more conformations of the enzyme, based on an analysis of the time course of proteolysis.

Materials and Methods

L-α-Phosphatidylcholine (type IX-E from egg yolk) was purchased from Sigma Chemical Co. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. [γ-^{32}P]ATP[1] (2–10 Ci/mmol) was purchased from New England Nuclear. Bovine pancreatic trypsin was purchased from Calbiochem. Bovine brain calmodulin

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[1] Abbreviations: TEA, triethanolamine; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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was prepared according to the method of Watterson et al. (1976) and stored at −20 °C. All other chemicals were of reagent grade.

Protein Purification. Except where stated otherwise, calmodulin-deficient human erythrocyte ghosts, prepared as described previously (Jarrett & Penniston, 1976), were solubilized at a protein concentration of 8 mg/mL in 0.5% Triton X-100, 300 mM KCl, 1 mM $MgCl_2$, 10 mM Tes–TEA, pH 7.4, 100 μM $CaCl_2$, and 2 mM dithiothreitol at 2 °C for 10 min. The suspension was centrifuged at 100000*g* at 2 °C for 40 min. A sonicated suspension of phosphatidylcholine (2% w/v) was added to the supernatant to a concentration of 1 mg/mL. The supernatant was then applied to a calmodulin–Sepharose 4B column that had been prepared according to the method of Klee & Krinks (1978) and that had been equilibrated with 0.5% Triton X-100, 300 mM KCl, 10 mM Tes–TEA, pH 7.4, 100 μM $CaCl_2$, 2 mM dithiothreitol, and 0.1% phosphatidylcholine. The column was washed with 0.05% Triton X-100, 300 mM KCl, 10 mM Tes–TEA, pH 7.4, 1–50 μM $CaCl_2$, 2 mM dithiothreitol, and 0.1% phosphatidylcholine for 18 h at a flow rate of 60 mL/h. The enzyme was eluted with the same medium containing 10 mM EDTA and no $CaCl_2$. The combined protein peak (monitored by measuring the absorbance at 278 nm) was stored in liquid nitrogen.

Ca^{2+}–ATPase Assay. ATPase activity was quantitated by measuring the liberation of inorganic phosphate from [γ-^{32}P]ATP (Jarrett & Penniston, 1977) at 37 °C and is expressed as micromoles of P_i released per milligram of protein per minute. The assays in Table I and in Figures 2 and 3 were done in triplicate for 30 min in media containing 5.0 mM EDTA, 25 mM Tes–TEA (pH 7.4 at 37 °C), 1.6 mM $CaCl_2$, 9.0 mM $MgCl_2$, 6.0 mM ATP, 3 μg of calmodulin (when called for), and 2–5 μg of purified ATPase. This mixture gave 0.34 mM free Mg^{2+}, 5.0 μM free Ca^{2+}, 28 μM $CaATP^{2-}$, and 5.2 mM $MgATP^{2-}$. Blanks obtained without any protein were subtracted from the activities.

Only for the experiment shown in Figure 2 was an optimal amount of Triton and lipid added. The assays were usually done with only the amount of lipid and Triton unavoidably present in the enzyme preparation.

Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) on 7.5% gels (7% gel for Figure 5). Gels were stained for protein with 0.2% Coomassie brilliant blue R-250 overnight and destained with methanol–acetic acid–water (5:1:5 v/v/v). The molecular weight of Ca^{2+}–ATPase was calculated by plotting log M_r vs. R_f for five standard proteins of known molecular weight, and fitting a straight line to these data. A modification of the procedure of Eckhardt et al. (1976) as described by Lutz et al. (1979) was used to detect glycoproteins. In some samples, the periodic acid–Schiff base stain (Zacharius et al., 1969) was employed.

$NaDodSO_4$ Pore Gradient Electrophoresis. Linear gradients with a gel concentration of 6–35% were prepared with the programmable LKB Ultrograd Gradient Maker, a Gilson peristaltic pump, and the Bio-Rad vertical slab gel system. Twelve protein standards ranging from 330000 to 14400 daltons were obtained from Pharmacia and Bio-Rad and dissolved at a concentration of 1 mg/mL in the following solubilizing buffer: 2% $NaDodSO_4$, 0.0625 M Tris, pH 6.8, 1% β-mercaptoethanol, 6 M urea, 0.002% bromophenol blue, and 10% glycerol. Purified ATPase was first precipitated with two volumes of 10% trichloroacetic acid and then resuspended in the solubilizing buffer and heated for 2 min in a boiling water bath. All samples were electrophoresed at 0 °C for 4.5 h at a constant power of 15 W/two slab gels. The tracking dye was allowed to run off the gel. Gels were stained with 0.2% Coomassie brilliant blue R-250 for 2 h, destained overnight, and placed in 10% glycerol for 90 min to restore the original shape of the gels; they were dried on a Bio-Rad gel slab dryer, Model 224.

Protein Determinations. Protein concentrations were determined according to the method of Lowry et al. (1951) modified by Bensadoun & Weinstein (1976) with bovine serum albumin as standard.

Amino Acid Analyses. Ca^{2+}–ATPase was purified as described above and dialyzed against 35% acetic acid for 5 days at 2 °C. The solution was lyophilized, and the dry protein powder was used for all subsequent amino acid analyses.

Amino acid analyses were carried out on a Beckman 121M autoanalyzer. All determinations were made in duplicate from 24-, 48-, and 72-h 6 N HCl hydrolysates to correct for destructive losses of serine, threonine, and proline and for the slow release of valine and isoleucine. Cysteine and methionine were determined as cysteic acid and methionine sulfone from 24-h 6 N HCl hydrolysates of performic acid oxidized Ca^{2+}–ATPase according to the method of Moore (1963). Tryptophan was determined after 24-h base hydrolysis.

We also carried out an amino acid analysis of the ATPase protein band of a 7.5% polyacrylamide gel. Ca^{2+}–ATPase (100 μg) was electrophoresed in the presence of $NaDodSO_4$ as described above. The gel was fixed and stained with Coomassie brilliant blue R-250 and washed extensively with 10% acetic acid. The band was excised, weighed, and lyophilized. The amino acid determination was made from a 24-h 6 N HCl hydrolysate of the dried gel. The determination was corrected for the values obtained with a blank gel treated identically.

Phosphate Determination. Ca^{2+}–ATPase was isolated as described above but in the absence of phosphatidylcholine and dialyzed extensively against 35% acetic acid, and its total phosphorus content was determined according to the method of Chen et al. (1956).

Partial Proteolysis of Purified Ca^{2+}–ATPase. Triton-solubilized ATPase (50 μL) (150 μg of protein/mL) was digested on ice for the times indicated with 1 μg of trypsin (1 mg/mL). The reaction was stopped by adding trypsin inhibitor (5 mg/mL) in 10-fold concentration excess. Aliquots of 20 μL were immediately tested spectrophotometrically for ATPase activity in a medium containing 50 μM $CaCl_2$, 120 mM KCl, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, 2.5 mM $MgCl_2$, 1 mM K-ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 IU of pyruvate kinase, and 1 IU of lactate dehydrogenase in a final volume of 1 mL at 37 °C. The difference in absorbance between 366 and 550 nm, measured with a dual wavelength spectrophotometer, was plotted vs. time. Subsequently, 50-μL aliquots were submitted to slab gel electrophoresis in 7% polyacrylamide (thickness of the gels 0.8 mm). The gels were stained by the silver impregnation method described by Merril et al. (1981).

Results

Isolation. The affinity chromatography elution profile of Triton X-100 solubilized ghosts was similar to that shown in Figure 7 of our previous report (Niggli et al., 1979) with the exception that the column was washed with a 1–50 μM Ca^{2+} buffer for 18 h before the enzyme was eluted with EDTA. The first peak contained over 90% of the solubilized ghost proteins, including the Mg^{2+}–ATPase, which was clearly separated from

Table I: Separation of Mg^{2+}-ATPase from Ca^{2+}-ATPase[a]

fraction	ATPase activity (nmol/mg·min) Mg^{2+}	Mg^{2+} and Ca^{2+}	Mg^{2+}, Ca^{2+}, and calmodulin
Ca^{2+} peak	6.0	0.6	1.1
EDTA peak	−2	189	496

[a] For the data in this table only, the sample was eluted from the affinity column with EGTA instead of EDTA. Similar results were obtained with EDTA elution. The total activities less the blank are shown. This table shows that (1) the Ca^{2+} peak contained no Ca^{2+}-stimulated ATPase (the inhibition by Ca^{2+} shown here was sometimes seen, but usually Ca^{2+} had little effect) and (2) the purified ATPase required Mg^{2+} but showed no activity in the presence of Mg^{2+} alone. The assay was carried out in a medium that was 6 mM in total ATP, 6 mM in total $MgCl_2$, 0.45 mM in free Mg^{2+}, and 0.27 μM in free Ca^{2+}. In order to achieve this, it was necessary to take account of the Ca^{2+} or EGTA present in the samples eluted from the column. The total concentrations of $CaCl_2$ and EGTA present, including that from the column buffers, were as follows: Ca^{2+} peak, 5.0 mM EGTA and 4.47 mM $CaCl_2$; EGTA peak, 6.19 mM EGTA and 5.5 mM $CaCl_2$. The Ca^{2+} peak contained a large amount of protein, which accounts for its low specific activity. The negative value of activity for the EDTA peak, in the presence of Mg^{2+} alone, reflects a negligible decrease below the blank that occurred when $MgCl_2$ was added.

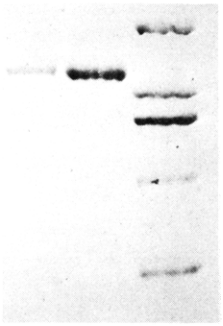

FIGURE 1: $NaDodSO_4$–polyacrylamide slab gel of Ca^{2+}-ATPase. Migration was from top to bottom. The right track shows molecular weight markers (200 000, 116 000, 93 000, 68 000, and 43 000, from top to bottom), and the middle and left tracks show 18 μg and 6 μg of ATPase, respectively.

the Ca^{2+}-ATPase (Table I). The Mg^{2+}-ATPase did not require Ca^{2+} and failed to respond to calmodulin, and its function is still unknown. This enzyme was missing in the second peak, which was composed entirely of Ca^{2+}-ATPase in a yield of approximately 0.1% of the initial ghost protein. Calmodulin activated the enzyme 10–20-fold at free Ca^{2+} concentrations in the vicinity of 1 μM. Figure 1 shows a $NaDodSO_4$–polyacrylamide slab gel of the pure Ca^{2+}-ATPase stained with Coomassie brilliant blue; no impurities were detected.

Chemical Characterization. When we electrophoresed different amounts of purified Ca^{2+}-ATPase on linear gradient polyacrylamide gels under denaturing conditions, no protein bands were visible in the region corresponding to the R_f value of calmodulin even when the gels were heavily overloaded. This suggests that calmodulin is not a subunit of functional Ca^{2+}-ATPase.

Using $NaDodSO_4$–polyacrylamide gel electrophoresis, we determined the molecular weight for Ca^{2+}-ATPase to be 138 000 ± 4000. This molecular weight is more precise than the value of about 125 000 previously reported from our laboratories (Niggli et al., 1979), due to a better choice of electrophoresis media. It agrees reasonably well with the previously reported value of 145 000 (Knauf et al., 1974; Wolf et al., 1977).

Table II: Amino Acid Composition of Ca^{2+}-ATPase

amino acid	mol/mol erythrocyte ATPase[a]	sarcoplasmic reticulum ATPase[b]
Lys	84	51
His	22	8
Arg	56	53
Asx	131	85
Thr	73	65
Ser	85	62
Glx	134	113
Pro	62	49
Gly	95	76
Ala	81	90
Cys	20	24
Val	97	86
Met	29	32
Ile	83	49
Leu	115	108
Tyr	26	23
Phe	49	44
Trp	7	[c]

[a] Based on a molecular weight of 138 000. [b] Based on a molecular weight of 102 000; taken from MacLennan et al. (1971). [c] Not determined.

Because of our previous observation of a significant amount of carbohydrates in a less pure bulk sample of the ATPase (Penniston et al., 1980), an extensive search was made to detect possible glycoprotein components in the pure ATPase. In the course of this study, eleven different samples of pure ATPase were subjected to electrophoresis and stained either according to the periodic acid–Schiff base technique or the dansylhydrazine method. No intensely staining bands were seen, and weakly staining components were seen only when the gels were loaded with very large amounts of protein. Consistent detection of weak bands that stained for sugar was possible only when 200 μg of pure ATPase was loaded onto a single gel tube and stained by the ultrasensitive dansylhydrazine method. When this was done, the ATPase band showed some nonspecific absorption of dansylhydrazine, which was also present in a control sample that had not been treated with periodate. Specific staining was not observed at the ATPase band but was seen at a point that corresponded to M_r 90 000; under the conditions utilized for our gels, glycophorin migrated at this position. The intensity of staining of this band corresponded to the staining of the glycophorin in 25 μg of ghosts. Assuming that about $^1/_{40}$th of ghost protein is glycophorin and that glycophorin is about half sugar, this would correspond to 0.3 μg of carbohydrate. On the basis of this estimate, sugars would constitute only about 0.15% of the total protein added.

It seems quite probable that the staining material is a small amount of glycophorin (less than 1% of the total sample) present as a contaminant. The material also might be band 3, the anion transport protein, but if so, it should also have been detectable by Coomassie blue staining. In any case, it seems clear that the pure, reconstitutable ATPase contained negligible amounts of periodate-cleavable carbohydrate, either as a part of the main polypeptide chain or as an associated polypeptide. This does not exclude the possibility that some glycoprotein may be associated with the enzyme in the membrane; however, the pure enzyme has most of the properties for the original enzyme and is devoid of such glycoproteins.

The amino acid compositions were determined from a bulk sample of purified Ca^{2+}-ATPase and from the 138 000-dalton band cut from a gel after electrophoresis; these were identical within experimental error. The composition of the bulk sample

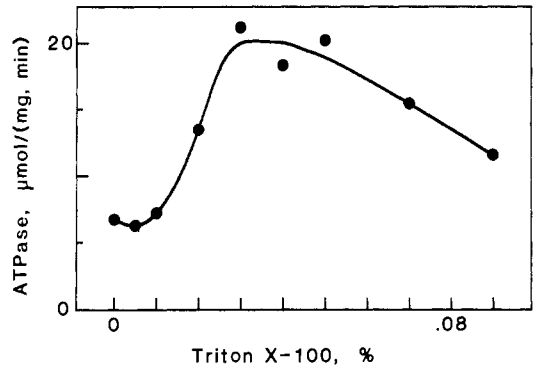

FIGURE 2: Effect of Triton X-100 in the assay medium on enzyme activity. The assay medium was 1.7 μg/mL in ATPase, 0.02% in asolectin, 40 mM in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, 5 mM in $MgCl_2$, 2 mM in ATP, 5 mM in EDTA, and 2 mM in $CaCl_2$ (free [Ca] 1.3 μM). The activity was measured by the coupled enzyme assay previously described (Niggli et al., 1981), and the necessary enzymes and substrates were also present in the medium. The ATPase utilized for this experiment was purified by a variation of the procedure described under Materials and Methods: the solubilization and purification media were all 0.2 M in sucrose, and asolectin was added instead of phosphatidylcholine. After the enzyme was loaded onto the column, the column was washed 3.5 h with 50 μM $CaCl_2$ and 1 h with 1 μM $CaCl_2$, and the enzyme was eluted with 0.25 mM EDTA; the other components of the media were as described under Materials and Methods.

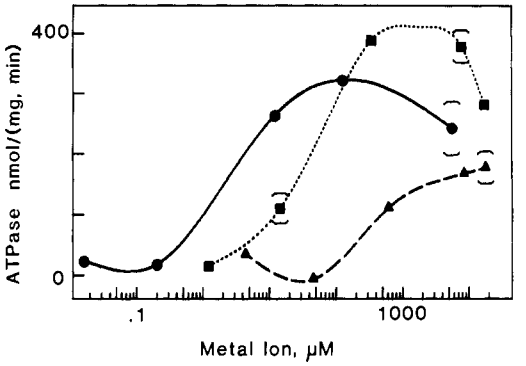

FIGURE 3: Stimulation of ATPase by Ca^{2+} (circles), Sr^{2+} (squares), and Ba^{2+} (triangles). Incubation was 30 min at 37 °C in a medium that was 50 mM in Tes–TEA, pH 7.4, 0.3 mM in EDTA, 2 mM in ATP, 2.5 mM in $MgCl_2$ and 0–10 mM in the alkaline earth chloride. The horizontal axis represents free ion concentrations that were calculated as previously described (Graf & Penniston, 1981) by using the following additional logarithms of association constants: BaATP, 3.29; BaH-ATP, 5.20; BaEDTA, 7.86; BaH-EDTA, 4.68; SrATP, 3.54; SrH-ATP, 5.15; SrEDTA, 8.73; SrH-EDTA, 4.04 (Martell & Smith, 1974; Smith & Martell, 1975). These constants were corrected for the activity of H^+ in water.

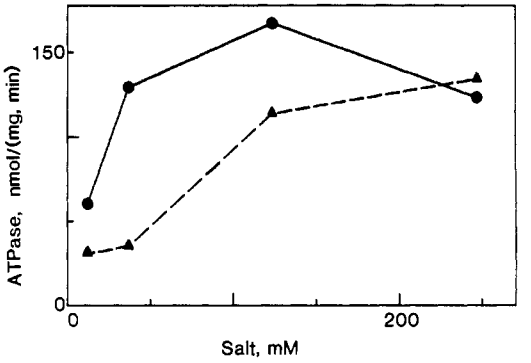

FIGURE 4: Stimulation of ATPase by Na^+ (circles) and K^+ (triangles). Incubation was 27 min at 37 °C in a medium containing 25 mM Tes–TEA, pH 7.4, 5.00 mM $MgCl_2$, 2.00 mM $CaCl_2$, 5.00 mM EDTA, 2.0 mM ATP, and 0.15 μg/mL calmodulin.

(extrapolated to zero hydrolysis time) is shown in Table II, together with comparative values for sarcoplasmic reticulum ATPase. Calculations of polarity by means of average hydrophobicity (Bigelow, 1967) and Barrantes' discriminant function (1975) showed that the Ca^{2+}–ATPase from ghost membranes lies on the borderline between typical integral membrane proteins and peripheral or soluble proteins. The average hydrophobicity of our ATPase was 1103 cal/mol, and the discriminant function is 0.29. Typical values of the discriminant function were as follows: for a group of nonmembrane proteins, 0.16 ± 0.11; for a group of integral membrane protiens, 0.52 ± 0.11 (Barrantes, 1975).

Phosphate analysis of the purified Ca^{2+}–ATPase detected 7 mol of P_i/mol of protein. The nature of the phosphorus in the enzyme is unknown; it may be tightly bound phospholipid or phosphorus covalently linked to amino acid residues.

Pure Ca^{2+}–ATPase showed a strong tendency toward aggregation. Incubation at temperatures above 30 °C, freezing and thawing, or even several weeks storage in liquid nitrogen caused the appearance (upon $NaDodSO_4$ gel electrophoresis) of dimers and higher aggregates. These aggregates were resistant to dissociation; even boiling in 2% $NaDodSO_4$, 1% β-mercaptoethanol, and 6 M urea failed to dissociate them. Similar aggregation properties of numerous other membrane proteins have been reported (Maddy, 1976). This observation suggests that Ca^{2+}–ATPase has one or several highly hydrophobic, exposed regions. The enzyme may be stored in liquid nitrogen with little loss of activity for several weeks; formation of the aggregates mentioned above was not associated with a loss of activity.

Effect of Assay Conditions on Observed Activity. The enzyme activity depended strongly on the amount of Triton and phospholipid in the assay medium. In the experiment shown in Figure 2, 0.02% asolectin was added to a typical assay medium, and the effect of varying the Triton concentration was studied. The addition of Triton to a concentration of 0.03–0.05% tripled the enzyme's activity.

Ion and Nucleotide Specificity. Figure 3 shows that Ca^{2+} was more effective than other alkaline earth metal ions in stimulating the ATPase. The rate attainable with Sr^{2+} was at least as great as that due to Ca^{2+}, but higher free Sr^{2+} concentrations were required to activate the enzyme. Ba^{2+} was considerably less effective than Sr^{2+}. Mg^{2+} was also ineffective in stimulating the ATPase, as is shown by the data in Table I. This ion specificity is consistent with the properties reported for the Ca^{2+} pump of red cells (Sarkadi, 1980) and the solubilized ATPase (Pfleger & Wolf, 1975).

The ATPase is highly specific for ATP as substrate; UTP, ITP, GTP, and CTP were all ineffective. The latter group gave, at most, 3.5% of the rate of hydrolysis given by ATP. This agrees with the substrate specificity shown for the enzyme in unfractionated membranes (Cha et al., 1971).

Effect of Na^+ and K^+. The ATPase in the presence of calmodulin was significantly stimulated by 100 mM Na^+ or K^+, as shown in Figure 4. A similar effect of Na^+ or K^+ was observed in the absence of calmodulin (data not shown). Previous work on unfractionated membranes has shown a similar effect of Na^+ or K^+ on Ca^{2+}–ATPase (Schatzmann & Rossi, 1971) and on Ca^{2+} uptake (Sarkadi et al., 1978).

Proteolysis. Trypsin-catalyzed hydrolysis for a short time (1 min) produced the complex initial fragmentation pattern shown in Figure 5. At least 14 proteolytic products were seen; the one having M_r equal to 40 000 stained more intensely than the others.

Incubation for longer times resulted in further cleavage of all the bands initially present. The 40 000-dalton fragment

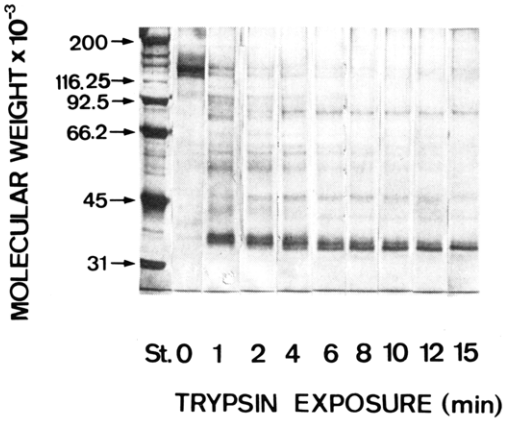

FIGURE 5: Proteolytic fragments from solubilized ATPase. See Materials and Methods for details.

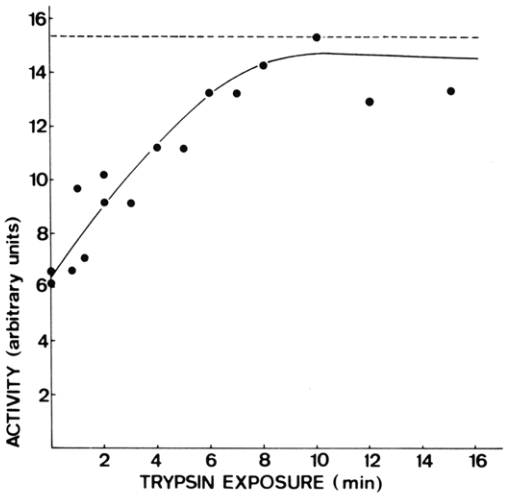

FIGURE 6: Activity vs. time of proteolysis for sample whose electrophoresis is shown in Figure 5. The dashed line shows the activity obtained with calmodulin, which had no effect on the activity after prolonged proteolysis.

was cleaved in two steps to a trypsin-resistant fragment of molecular weight 33 500. The initial fragments of 110 000, 98 000, and 91 000 gave rise to another trypsin-resistant fragment that migrated at an apparent molecular weight of 81 000.

The data clearly argue for more than one form of the initial enzyme; presumably the different forms are different conformations of the 138 000-dalton chain of the ATPase. A number of lines of reasoning are possible, but the simplest one is to point out that in some molecules, the 81 000-dalton fragment was resistant to trypsinization, while others were immediately cleaved into fragments of intermediate sizes. These intermediate fragments had apparent molecular weights of 77 000, 76 000, 66 000, 57 000, and 52 000. These fragments can only have been produced by trypsin cleavage at a site that is within the 81 000-dalton fragment. Some of the molecules of the ATPase were evidently folded in such a manner as to prevent trypsin cleavage within this 81 000-dalton fragment, while others were immediately cleaved at a point within this fragment. This latter group of molecules was then further degraded. Their degradation yielded the 46 000-dalton intermediate (which was prominent at times between 4 and 10 min) and ultimately gave much smaller fragments. Such behavior is consistent with the evidence for aggregation and other complex behavior of this large, membrane-spanning peptide.

Of all of the proteolytic products produced, only two have a time course consistent with their being responsible for the increase in enzyme activity shown in Figure 6: The enzyme activity was about half stimulated at 4 min, and this is consistent with the time at which the 81 000- and 33 500-dalton bands appeared. The observation that a relatively high molecular weight intermediate can be phosphorylated in trypsinized erythrocyte membrane vesicles (Enyedi et al., 1980) and our own preliminary observations indicate that the 81 000-dalton fragment is the activated ATPase.

Discussion

The above results demonstrate that the minor impurities, present in our original preparation (Niggli et al., 1979), can be removed by extensive washing of the enzyme while it is bound to the calmodulin–Sepharose column. The isolated enzyme retains high enzymatic activity and is responsive to calmodulin. The present enzyme preparation is virtually free of band 3, a protein that is many times more abundant than Ca^{2+}-ATPase and that copurified in previous isolations.

The most plausible explanation for the improved purity achieved by the 18-h wash is the presence of micelles containing both Ca^{2+}-ATPase and contaminating proteins. Each micelle is attached to the column through an ATPase molecule. As micelles containing no protein are passed over the column, there is probably a slow exchange of the contaminating protein molecules between bound micelles and mobile micelles, allowing the removal of the contaminants.

We did not calculate the degree of purification for the following reasons: (1) Inactivation occurred during the isolation; because of this, a calculated degree of purification would be only a minimum value. (2) The activity of the Ca^{2+}-ATPase is regulated by many factors. Some of these are not controlled in the preparation of erythrocyte ghosts, as is shown by the great variability in specific activity of the Ca^{2+}-ATPase from one preparation of ghosts to another. Thus, the assigned percent recovery of enzymatic activity becomes somewhat arbitrary and ranges between 10% and 120% of the solubilizate depending on the pCa of the assay medium and the presence or absence of calmodulin.

As discussed under Results, the 138 000-dalton band contains no stainable carbohydrate. The appreciable amount (about 7%) of carbohydrate detected by bulk analysis of an earlier Ca^{2+}-ATPase preparation (Penniston et al., 1980) was probably caused by a greater glycophorin contamination of that sample, which was not washed extensively to remove impurities.

The high polarity of this protein, relative to that of sarcoplasmic reticulum ATPase or the integral membrane proteins analyzed by Barrantes (1975), can probably be explained in terms of its high molecular weight. The sample of integral membrane proteins studied by Barrantes was heavily weighted toward low molecular weight proteins such as the acetylcholine receptor (about 40 000) and rhodopsin (about 36 000). Small proteins such as these would necessarily have a larger proportion of their surface in contact with the hydrophobic region of the membrane. A very large protein such as the Ca^{2+}-ATPase studied here could provide an equal or greater area for membrane contacts and still have a larger proportion of hydrophilic regions, both on the inside and on the outside of the membrane.

The studies of the enzymic properties of the pure ATPase show that it shares to a considerable extent the properties ascribed to the Ca^{2+}-ATPase in the unfractionated erythrocyte membrane. Even in the solubilized form, the behavior of the purified enzyme is qualitatively the same as the original. Previous work from our laboratories (Niggli et al., 1981a,b)

showed that the dependence of activity on pCa and the responses to calmodulin and vanadate were not qualitatively different from these properties in the intact enzyme. This report adds the ion and nucleotide specificity and monovalent ion responsiveness to the list of properties retained by the pure enzyme.

As analyzed under Results, the proteolysis data indicate the presence of more than one conformation of the solubilized ATPase. This is consistent with our other experience with purified enzyme, particularly the observation that it is slowly inactivated upon standing at 4 °C (data not shown). It appears probable that the inactive conformation of the enzyme exposes more sites for typsin cleavage and gives rise to the products of intermediate molecular weight that are not associated with the increase in enzyme activity. This analysis suggests that the active form of the enzyme contains an 81 000-dalton polypeptide chain that is folded in such a manner as to protect its residues from trypsin cleavage.

A recent study (Stieger & Schatzmann, 1981) has reported that two fragments were produced upon trypsinization of the purified, solubilized ATPase. However, the sensitivity and resolution of their electrophoresis was not sufficient to detect the many bands observed here.

Previous work in our laboratories has achieved a specific activity at 37 °C for human erythrocyte Ca^{2+}-ATPase of 6–7 μmol/(mg·min) (Niggli et al., 1981a,b), while others have reported a value of 10.1 μmol/(mg·min) (Gietzen et al., 1980). The results shown in Figure 2 show that our previous measurements of activity have underestimated the catalytic capability of purified enzyme. Our previous assay method, used in all other figures of this paper and in several previous papers (Carafoli et al., 1980; Niggli et al., 1979, 1981a,b; Graf & Penniston, 1981), involved dilution of the enzyme with an aqueous medium containing no Triton or phospholipid. When the enzyme used for Figure 2 was assayed in this way, it gave an activity of 5.0 μmol/(mg·min), comparable to the activity shown in the figure without added Triton.

It should be noted that the system required for these assays (Triton X-100–phospholipid–water–protein) is a complex one. In particular, in the absence of Triton, the phospholipid forms a highly turbid suspension; in our experience, the amount of Triton X-100 required for maximal activation was the same as that required to make the solution nearly clear. The concentration of Triton on a weight basis should be about twice that of phospholipid, in order to achieve this. It thus appears that the relatively low activity observed at low Triton concentrations was caused by the occurrence of large phospholipid aggregates. The protein concentration may also affect the activation, although we have not explored this variable.

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