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Isolation and Characterization of Erythrocyte Membrane Ca^{2+}-ATPase in Cystic Fibrosis

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ABSTRACT. Ca^{2+}-ATPase was purified from erythrocyte membranes prepared from cystic fibrosis (CF) blood samples (n = 10) and from age/sex-matched control blood samples (n = 10). The kinetics of calcium activation of the purified enzyme was investigated in the presence of asolectin phospholipids and found to be virtually identical for both CF and control preparations: V_{Ca}^{2+} = 3.01 ± 0.24 μmol ATP hydrolyzed/mg pure enzyme/min (mean ± SE) and 3.09 ± 0.20 for CF and control Ca^{2+}-ATPase, respectively; K_{Ca}^{2+} = 0.328 ± 0.046 μmolar free calcium and 0.333 ± 0.040 for CF and control enzyme, respectively. The preparative procedure used (one-step purification by calmodulin-affinity chromatography) allowed quantitative recovery of essentially 100% of the Ca^{2+}-ATPase present in detergent-solubilized erythrocyte membranes, enabling expression of the yield of purified enzyme in terms of the quantity of starting membrane protein: 0.127% ± 0.006% (w/w) and 0.140% ± 0.007% for CF and control enzyme preparations, respectively. None of the parameters evaluated showed a statistically significant difference ($p < 0.05$) between the CF and control groups. Furthermore, when CF and control purified Ca^{2+}-ATPase samples were analyzed by high-resolution gradient SDS-polyacrylamide gel electrophoresis, no differences in mobility were observed (mol wt = 128 kdaltons). Thus, Ca^{2+}-ATPase purified from CF erythrocyte membranes and assayed in the presence of asolectin appears to be quantitatively similar to control purified enzyme in amount, molecular weight, and kinetics of activation by calcium. These data suggest that Ca^{2+}-ATPase may not be the defective gene product in CF. Previous reports of deficient Ca^{2+}-ATPase hydrolytic and transport activities observed *in situ* within CF plasma membranes may reflect an abnormality of membrane components in close association with the Ca^{2+}-ATPase (*e.g.* lipid or protein activators or inhibitors). (*Pediatr Res* 20: 356–360, 1986)

Abbreviations

CF, cystic fibrosis
SDS, sodium dodecyl sulfate

CF is a genetic exocrinopathy of unknown etiology (1). There is good evidence that alteration of cellular calcium homeostasis may play a major role in the pathogenesis of this disorder. However, it is unclear at present whether the cellular disturbance in calcium handling is the primary defect of CF (2).

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Work performed in our laboratory (3–7), as well as studies conducted by other investigators (8–13), indicated that the activity of the calcium-transporting ATPase enzyme (Ca^{2+}-ATPase) of CF erythrocyte and fibroblast plasma membranes is deficient. We found that the maximal calcium activation (V_{Ca}^{2+}) of $^{45}Ca^{2+}$-transport and ATP hydrolysis of CF membranes is approximately 50% of control levels. However, the affinity of these processes for calcium ion (1/Kd) is similar in CF and control membranes (3–7). The exact relationship between these observations and the evidence for disturbances in cellular calcium homeostasis, particularly in exocrine glands, remains to be elucidated (2).

To determine whether this Ca^{2+}-ATPase deficiency reflects the basic CF defect necessitates the characterization of the enzyme in purified form. The present report presents data on the partial characterization of purified CF Ca^{2+}-ATPase. A preliminary report of this work has been presented (29).

MATERIALS AND METHODS

Materials. Asolectin (AX1778) was purchased from MCB Reagents Company. The following materials were purchased from Sigma Chemical Company: Calmodulin-Agarose (p4385), Tween 20, Triton X-100, EDTA, CDTA (trans-1,2-diaminocyclohexanetetraacetic acid), adenosine 5′-triphosphate from equine muscle (tris salt), and bovine albumin (fraction V, A4503). The following materials were obtained from Fisher Scientific Company: carbon decolorizing alkaline (Norit A) (activated charcoal), and high pressure liquid chromatography-grade acetonitrile. Sodium dodecyl sulphate was supplied by BDH Chemicals Ltd. and Fluram (fluorescamine) by Roche Diagnostics. Gamma-^{32}P-adenosine 5′-triphosphate was purchased from Amersham Corporation. Aquasol LSC cocktail was supplied by New England Nuclear Company. All other chemicals used were of reagent grade or better. Deionized distilled water was used exclusively in these studies.

Subjects. Following informed consent, blood samples were drawn from 10 CF patients (mean age = 25.6 yr; age range: 19–41 yr; four females and six males) and from 10 age/sex-matched control volunteers (mean age = 26.3 yr; age range: 20–42 yr). Two 7-ml EDTA-anticoagulated tubes of blood were collected from each subject.

Preparation of erythrocyte membranes. Calmodulin-deficient erythrocyte membranes were prepared at 4° C by a modification of the method of Niggli *et al.* (14): each whole blood sample (10–14 ml) was washed three times in 5 volumes of 130 mM KCl, 20 mM Tris-Cl, pH 7.4, and centrifuged at 2500 × g to remove plasma and the buffy coat. Washed erythrocytes were then lysed in 5 volumes of 1 mM EDTA-containing 10 mM Tris-Cl, pH 7.4 and centrifuged 10 min at 35,000 × g. Membranes were washed five times in this hemolysis solution with intervening stirring (10 min, 4° C) and then four times in 10 mM Hepes, pH 7.4. Finally, erythrocyte membranes were resuspended in a 20 mM Hepes, pH 7.4 storage solution containing 130 mM KCl, 500 μM $MgCl_2$, 50 μM $CaCl_2$, and 2 mM dithiothreitol. Mem-

branes were then quick frozen and stored at −80° C until use (10 days maximum storage time). Twenty to 30 mg of membrane protein, as determined by the Lowry protein assay (15), could be produced by this method.

Purification of erythrocyte membrane Ca^{2+}-ATPase by calmodulin affinity chromatography. Ca^{2+}-ATPase was purified from CF and control erythrocyte membranes by modification of the methods of Penniston and Carafoli and their colleagues (14, 16). Calmodulin-deficient erythrocyte membranes (20–30 mg) were centrifuged at 100,000 × *g* at 4° C for 5 min, and the pellet was solubilized at 4° C for 10 min at a protein concentration of 5 mg/ml in a solubilization solution somewhat similar to that used by Graf *et al.* (16): 10 mM Hepes, pH 7.4 containing 0.45% Triton X-100 (w/v), 0.05% Tween 20 (w/v), 300 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, and 2 mM dithiothreitol. The solubilized membranes were then centrifuged at 100,000 × *g* at 4° C for 30 min. The ultra-high speed supernatant was not treated with a sonicated phospholipid suspension, nor was such a suspension added to subsequent chromatography media, as others have done (14, 16). Such treatment would have interfered with the protein estimation of the final purified product. Instead, the untreated supernatant was added directly to a 15 × 125 mm capped polypropylene tube containing 2.0 ml calmodulin-agarose pre-equilibrated with the solubilization solution. The tube was then topped up with solubilization solution and capped, and the solubilized Ca^{2+}-ATPase was allowed to bind to the immobilized calmodulin during 20 min of end-over-end mixing at 4° C. Following binding, each 2-ml aliquot of gel was poured onto a small chromatography column (BioRad Econo-column, 5 × 100 mm) and was exhaustively washed (100–200 × V_t) for 3 h at 4° C with 10 mM Hepes, pH 7.4 containing 0.05% Tween 20 (w/v), 300 mM KCl, 2 mM dithiothreitol, and 50 μM $CaCl_2$. The purified Ca^{2+}-ATPase was then eluted from the calmodulin-agarose gel with 4 ml of the same washing buffer also containing 2 mM EDTA. The effluent was made 2 mM with respect to $CaCl_2$ to "neutralize" the chelator. Each purified enzyme preparation was then aliquoted and stored until use: calcium activation kinetics of each preparation were performed within 2 h of elution on 4° C stored aliquots. Protein determinations and electrophoretic analysis were performed on aliquots stored at −80° C; these aliquots could be stored for up to several weeks without significant degradation.

Analysis of calcium activation kinetics of purified Ca^{2+}-ATPase preparations. A radiometric Ca^{2+}-ATPase assay was used to characterize the activity of the purified enzyme preparations based on ^{32}Pi released from γ ^{32}P-ATP (17). The modified method may be summarized as follows: 20-μl aliquots of purified Ca^{2+}-ATPase were assayed in 1.5-ml plastic centrifuge tubes at 37° C for 20 min in a final reaction volume of 600 μl, containing 40 mM Hepes, pH 7.4, 200 μM Tris-ATP, 1 mM Mg Acetate, 120 mM KCl, 130 μM EGTA, 100 μM CDTA, 100 μM ouabain, 2 mM dithiothreitol, 0.05 Triton X-100 (w/v), 0.02% sonicated asolectin (a mixture of soybean phospholipids rich in acidic species) (w/v), 400,000 dpm γ ^{32}P-ATP and varying amounts of total $CaCl_2$ (80, 100, 110, 120, 130, 140, 160, and 180 μM), corresponding to 0.086, 0.175, 0.270, 0.413, 0.680, 1.08, 2.48, and 5.03 μM free calcium, respectively. Free calcium concentrations were determined using Goldstein's Fortran program, CATIONS (18). Equilibrium constants for cations and ligands were obtained from Martell and Smith (19) and were corrected for ionic strength, pH, and temperature according to the methods described by these authors. Equilibrium constants for monoprotonated species were calculated according to the procedure of Blinks *et al.* (20). The total calcium values shown included two sources of "contaminant" calcium: 68 μM calcium/20 μl purified enzyme arising from EDTA-neutralization of enzyme solutions eluted from the calmodulin-agarose and 10 μM calcium/20 μl purified enzyme arising cumulatively from trace calcium contaminants in the various buffers and salts used in the preparation. The extent of this latter calcium source was determined by atomic absorption spectrometry. The Ca^{2+}-ATPase reaction as described above was terminated by addition of 100 μl cold 40% (v/v) trichloroacetic acid, containing 5 mM Tris-ATP and 2 mM KH_2PO_4. Five hundred μl of a charcoal suspension (0.1 g/ml in 5% trichloroacetic acid) was added to each tube. Tubes were shaken continuously for 5 min (Eppendorf Shaker 5432) and centrifuged for 5 min (Eppendorf Microfuge). Six hundred-μl aliquots of the supernatant were counted in 5 ml Aquasol scintillation fluid. The kinetics of calcium activation of purified Ca^{2+}-ATPase solutions were determined by Michaelis-Menton analysis and $V_{Ca^{2+}}$ and $K_{Ca^{2+}}$ were obtained from Eadie-Hofstee plots.

Determination of protein content of purified enzyme preparations. Protein contents of enzyme solutions were assayed by an ultramicro modification of the fluorescamine method of Castell *et al.* (21). In this assay, 20-μl aliquots of purified enzyme or 20-μl aliquots of protein standards (0–500 ng bovine serum albumin dissolved in calmodulin-agarose column washing buffer containing 2 mM $CaCl_2$ and 2 mM EDTA) were dispensed into 1.5-ml plastic centrifuge tubes in an ice bath. Seventy μl cold 0.2 M sodium borate buffer, pH 9.0, were added to the tubes. The contents of the tubes were vortexed and incubated at 4° C for a minimum of 5 min. Thirty μl 0.03% fluorescamine (w/v in acetonitrile) were added to each tube to begin the primary amino group-labeling reaction. The addition of fluorescamine was effected over a period of 10 s in small aliquots (about 6 μl each) using a positive-displacement analytical micropipet (SMI) with intermittent bursts of vortexing; following addition of the final aliquot of fluorescamine, each tube was vortexed continuously for 20 s and transferred to a rack to incubate at room temperature. To ensure that the time between fluorescamine addition and reading of the fluorescent signal was constant for all tubes, labeling reactions were initiated at 90-s intervals, and each tube was read after exactly 20 min incubation. The fluorescence of each sample was read by aspiration of the entire 120 μl reaction mixture into the flow cell of a modified Waters Model 420 Fluorescence Detector (7 μl sensed volume), equipped with a 395 nm excitation filter and a 460 nm emission filter. The fluorescence developed in each tube was proportional to the primary amino titer of each sample, which in the case of homogeneous protein solutions reflects its protein content. The amount of Ca^{2+}-ATPase in each purified enzyme preparation was read from the bovine serum albumin standard curve. Protein contents of Ca^{2+}-ATPase preparations determined by this method correlated well with those determined using the DOC-trichloroacetic acid precipitation modification (22) of the Lowry protein assay (when both assays were calibrated with bovine serum albumin protein standards: 1.0 μg Lowry protein = 1.1 μg fluorescamine protein ($r = 0.995$).

High resolution SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in 1.5-mm thick slab gels according to a modification of the method of Laemmli (23): the sample buffer was identical to the 0.0625 M Tris-HCl, pH 6.8 buffer used by this worker; the stacking gel was 5% acrylamide, 0.1% (w/v) SDS and 0.315 M Tris-HCl, pH 6.8; the separating gel was a 5–20% (w/v) acrylamide and a 0.13-1.0% (w/v) bis-acrylamide gradient in 0.375 M Tris-HCl, pH 8.8 and 0.1% SDS. Following electrophoresis at 15 ma/slab for 16 h, gels were either fixed and stained according to the silver method of Morrissey (24), or with 0.25% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid, and destained overnight in 7.5% (v/v) acetic acid/5% (v/v) methanol. Blue gels were analyzed by densitometry at 595 nm to assess the purity of Ca^{2+}-ATPase preparations.

Statistical analysis. The statistical significance of the differences of the means of parameters determined for CF and control purified Ca^{2+}-ATPase preparations was assessed using Student's *t* test for independent samples.

RESULTS

Fluorescamine ultramicro protein assay. Bovine serum albumin standard curves for individual fluorescamine assays were found to be linear from 0–500 ng (r = 0.995 or better), and assays were sensitive down to 25 ng protein (data not shown). CF and control purified Ca^{2+}-ATPase preparations contained roughly 175 ng/20 μl and fell well within the optimal protein range for the assay.

Calcium activation kinetics of CF and control purified Ca^{2+}-ATPase. Figure 1 shows the pooled calcium activation data for CF (n = 10) and for control (n = 10) purified enzyme preparations. It is clear from this graphical presentation that the CF and control Ca^{2+}-ATPase samples did not differ with respect to their kinetics of activation by calcium. This conclusion is borne out by examination of Table 1, which presents the mean ± SE data for kinetic parameters derived from individual Eadie-Hofstee plots for each CF or control purified Ca^{2+}-ATPase preparation. There is no statistically significant difference between CF and control data pools in these kinetic parameters.

When ATPase assays were conducted in the presence of 1 mM EGTA, effectively producing 0 μM free Ca^{2+} conditions, ATP hydrolysis corresponded to levels observed in assay "blanks" reacted in the absence of membrane protein (data not shown). This indicated that both CF and control purified Ca^{2+}-ATPase preparations were devoid of Mg^{2+}-ATPase activity.

Assessment of the content of Ca^{2+}-ATPase of CF and control erythrocyte membranes. The calmodulin-affinity chromatography method described above for the purification of Ca^{2+}-ATPase from erythrocyte membranes provides a molar excess of Ca^{2+}-ATPase binding sites (at approximately 50 μg ATPase bound/2 ml of calmodulin-agarose gel) compared with the amount of available Ca^{2+}-ATPase contained in 20–30 mg solubilized erythrocyte membrane protein (*i.e.* 20–35 μg purified enzyme). This single-step chromatographic purification technique allows virtually 100% recovery of the solubilized Ca^{2+}-ATPase under these conditions, for doubling or tripling the amount of calmodulin-agarose gel used resulted in no increase in the quantity or activity of the purified Ca^{2+}-ATPase recovered from the column (data not shown). Similarly, doubling or tripling the volume of eluent collected for a given amount of affinity gel similarly produced no increase in enzyme recovered (data not shown). Therefore, this technique enables the estimation of the titer of the enzyme in the original membrane preparation. After correction for 16% protein contamination of purified enzyme preparations (determined by densitometry of SDS-polyacrylamide electrophoretic

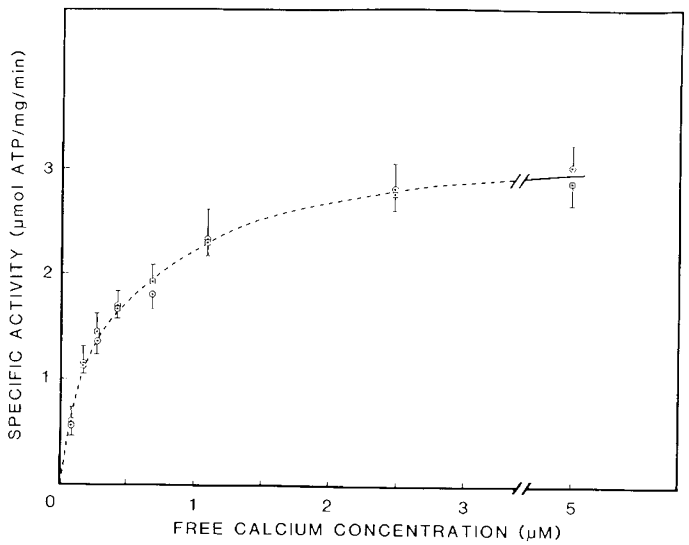

Fig. 1. Calcium activation curve for CF (n = 10) and control (n = 10) purified Ca^{2+}-ATPase preparations. In this graph, individual enzymatic activities have been pooled to permit display of mean values and SE *bars* for each calcium concentration assayed. Pooled CF data are designated by *open boxes* and pooled control data by *open circles*.

Table 1. *Calcium activation kinetics of purified Ca^{2+}-ATPase*

Subject	n	$V_{Ca^{2+}}$ (μmol/mg/min)	$K_{Ca^{2+}}$ (μMolar free Ca^{2+})
CF	10	3.01 ± 0.24*	0.328 ± 0.046
Control	10	3.09 ± 0.20	0.333 ± 0.040

* Mean ± SE.

Table 2. *Ca^{2+}-ATPase content of erythrocyte membranes*

Subject	n	Quantity of Ca^{2+}-ATPase (expressed as a percentage of total membrane protein)
CF	10	0.127% ± 0.006%*
Control	10	0.140% ± 0.007%

* Mean ± SE.

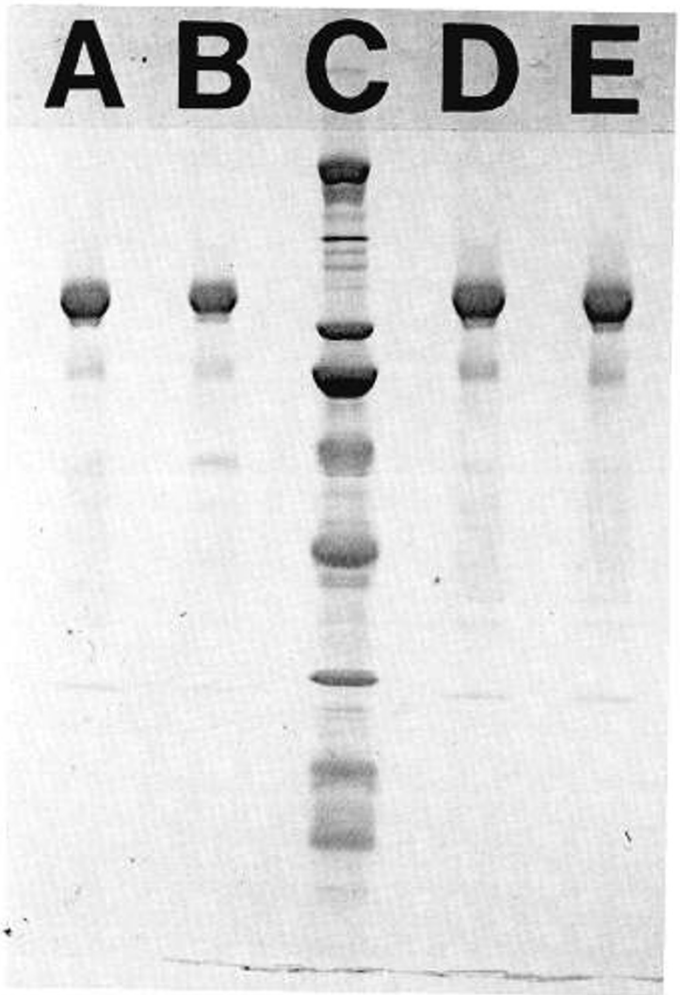

Fig. 2. High-resolution SDS-polyacrylamide gel electrophoresis of representative CF (*tracks B and E*) and control (*tracks A and D*) purified Ca^{2+}-ATPase preparations. The center profile (*track C*) shows BioRad molecular weight marker proteins (and smaller amounts of contaminating protein species): (from top to bottom) myosin (200 kd), seven intervening contaminating proteins, β-galactosidase (116.3 kd), phosphorylase B (92.5 kd) bovine serum albumin (66.2 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd).

gels), total yields of Ca^{2+}-ATPase were expressed as a percentage of starting membrane protein for each preparation. Table 2 presents the mean (± SE) protein yields of purified CF and control Ca^{2+}-ATPase in terms of the quantity of starting membrane protein: CF Ca^{2+}-ATPase was found to represent a similar percentage of the total membrane protein as control Ca^{2+}-ATPase.

SDS-polyacrylamide gel electrophoretic analysis of purified enzyme preparations. Figure 2 shows the electrophoretic profiles of two CF (tracks B and E) and two age/sex-matched control (tracks A and D) purified Ca^{2+}-ATPase preparations. Track C shows the banding pattern for a mixture of BioRad high and low molecular weight marker proteins (and trace contaminants). Densitometric analysis of Coomassie Blue stained gels showed the purity of both CF and control enzyme preparations to be 84% (purity range 81–88%). The molecular weight of CF and control Ca^{2+}-ATPase was identical: 128 kdaltons (see Fig. 2).

DISCUSSION

A number of investigators have suggested that a defect in cellular calcium homeostasis may be the etiologic principle, or at least may constitute an important element in the pathogenesis, of CF (2). Work in our laboratory (3–7) and that performed by a number of other independent investigators (8–13) has indicated that the transport and hydrolytic activity of plasma membrane-bound Ca^{2+}-ATPase of erythrocytes and fibroblasts appears to be deficient in CF. If a similar deficiency is found to occur in CF exocrine cells, this may explain a number of aspects of secretory and respiratory pathophysiology found to occur in this disorder (2). The possibility that plasma membrane-bound Ca^{2+}-ATPase could represent the defective gene product in CF suggests the importance of physical and enzymologic characterization of CF and age/sex-matched control Ca^{2+}-ATPase purified from calmodulin depleted erythrocyte membranes.

Methods were developed in our laboratory which permitted the microscale isolation and characterization of purified erythrocyte membrane Ca^{2+}-ATPase from 10- to 14-ml samples of whole blood. The isolation procedure differed in three major ways from the standard method of Niggli *et al.* (14): the reduction of the scale of the purification procedure, the omission of sonicated suspensions of stabilizing exogenous phospholipids from isolation and storage media, and the substitution of the detergent Tween 20 for Triton X-100 during washing and elution stages of calmodulin-affinity chromatography. Similar to the preparation of Niggli *et al.* (14), purified Ca^{2+}-ATPase made by this procedure was found to be devoid of Mg^{2+}-ATPase activity (see "Results").

This current study indicates that the kinetics of calcium activation of purified CF and control Ca^{2+}-ATPase appear identical. This result is borne out when mean data for $V_{Ca}{}^{2+}$ and $K_{Ca}{}^{2+}$ are compared for CF and control preparations (see Table 1). It should be pointed out, however, that these data do not unequivocally rule out the possibility of a molecular defect within the CF Ca^{2+}-ATPase, as they have been generated under special conditions: using solubilized Ca^{2+}-ATPase activated by calcium in the presence of asolectin, a mixture of soybean phospholipids rich in acidic species known to produce maximal stimulation of the enzyme (25). It is possible that a subtle molecular defect in CF Ca^{2+}-ATPase may be masked under these conditions and may only be manifest upon exposure to endogenous lipids or to exogenous lipids producing submaximal stimulation. Similarly, if the defect lies within molecular structures of the enzyme responsible for proper membrane polarity and alignment or those responsible for interfacing with membrane regulatory mechanisms, elucidation of the defect becomes more difficult and might only be accomplished by extensive reconstitution studies using highly defined proteoliposomes or by complete sequential analysis of the primary structure of the enzyme.

The results of the electrophoretic analysis of CF and control purified Ca^{2+}-ATPase preparations (see Fig. 2) indicate that there are no gross structural defects within the CF enzyme affecting its molecular weight; both CF and control preparations exhibited very similar molecular weights (approximately 128 kdaltons). These estimates are 8% lower than those found by Graf *et al.* (16) for Ca^{2+}-ATPase purified from erythrocytes of normal subjects (*i.e.* 138 kdaltons). This small difference between our preparations and those of Graf *et al.* (16) probably reflects differences in the resolving capabilities of our respective polyacrylamide gel systems. Graf *et al.* (16) used a 7.5% homogeneous gel. Our own high-resolution system (*i.e.* 5–20% acrylamide/0.13–1.0% bisacrylamide parallel gradients) produces good protein sieving, enabling compact banding and easy visualization of even trace amounts of proteins. This can be seen in the number of detectable protein contaminants found in the protein molecular weight calibration markers (see track C, Fig. 2). Also, this system permits an accurate estimation of contaminating proteins within the purified Ca^{2+}-ATPase preparations themselves.

Miner *et al.* (26), on the basis of phosphorylation studies of CF and control erythrocyte membranes, have suggested that CF membranes have approximately half the number of Ca^{2+}-ATPase sites as control membranes. Further, these workers have suggested that this might explain the deficient Ca^{2+}-ATPase activity reported by others (3–13). However, measurement of the steady-state Ca^{2+}-dependent phosphorylation of CF and control erythrocyte membranes by Waller *et al.* (27) revealed no significant difference between these two groups, indicating similar numbers of enzyme molecules per cell. By using a molar excess of Ca^{2+}-ATPase binding sites and designing our experiments to permit quantitative elution and assay of solubilized Ca^{2+}-ATPase, we have been able to estimate the Ca^{2+}-ATPase content of CF and control erythrocyte membranes. These have been found to be similar when expressed as a percentage of total membrane protein (see Table 2). Differences in CF and control phosphorylation patterns therefore probably reflect other membrane differences between these two groups of individuals.

The deficiency of CF erythrocyte membrane Ca^{2+}-ATPase activity previously noted (3, 5, 8–13) may reflect an abnormality of membrane components in close association with the ATPase enzyme (*e.g.* lipids or protein activators or inhibitors). Alterations in the lipid composition of CF membranes have been reported (28). It would be of interest therefore to conduct Ca^{2+}-ATPase activation studies with lipids extracted from CF erythrocyte membranes.

In conclusion, partial characterization of CF and control purified Ca^{2+}-ATPases has revealed no significant differences: the enzyme purified from CF erythrocyte membranes and assayed in the presence of asolectin phospholipids appears quantitatively similar to age/sex-matched control Ca^{2+}-ATPase in amount, molecular weight, and calcium activation kinetics. Further enzymologic studies of soluble and reconstituted CF erythrocyte Ca^{2+}-ATPase in the presence of other membrane regulatory components are warranted before finally concluding that the enzyme is not the defective gene product in CF.

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