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PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF A LOW-AFFINITY Ca^{2+}-ATPase FROM ERYTHROCYTE MEMBRANES

STELLAN HJERTÉN * and HUAZHEN PAN **

Institute of Biochemistry, University of Uppsala, Biomedical Center, Box 576, S-751 23 Uppsala (Sweden)

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A low-affinity Ca^{2+}-ATPase from erythrocyte membranes has been purified by agarose suspension electrophoresis and polyacrylamide gel electrophoresis in the absence of detergents. For maximal activity a calcium concentration above 10 mM is required. The activity is independent of magnesium. The K_m value for ATP is about 60 μM. The enzyme appears in two forms (A and B) with similar amino acid composition. The specific activity of A is higher than that of B. Gel electrophoresis in SDS of A gives a pattern consisting of two bands. B gives the same pattern; the only difference between the patterns is the ratio of the amounts of protein in the bands. The apparent molecular weight of the proteins in the two SDS bands has been estimated at 23000 and 21000, respectively. The results obtained can be explained by assuming that the two proteins corresponding to the two bands obtained in SDS electrophoresis have a similar structure and can associate to complexes A and B. We have also shown that electrophoretic and chromatographic supporting media can induce aggregation of (membrane) proteins. Artificial complexes can thus be formed and cause misinterpretation of the data obtained. This may be the reason why some authors have speculated that Ca^{2+}-ATPase is active only in complex with other proteins such as spectrin and actin .

Introduction

Erythrocyte membranes exhibit two Ca^{2+}-ATPase components, which differ with respect to their affinities for calcium ions. One is called low-affinity Ca^{2+}-ATPase, which shows a calcium-enzyme dissociation constant in the mM range. It is often considered to be responsible for the shape and deformability of the erythrocyte membrane [1]. The other, called high-affinity Ca^{2+}-ATPase, has a calcium-enzyme dissociation constant of about 1 μM, and is responsible for the active outward transport of Ca^{2+} across the membrane to maintain the very steep calcium ion concentration gradient between the inside and outside of the erythrocyte membrane. Avissar et al. [2] have reported the presence of Ca^{2+}-ATPase activity in a preparation obtained by molecular-sieving on Sepharose 4B and containing spectrin and three other proteins of molecular weights 80000, 60000 and 46000. White and Ralston [3] have described a Ca^{2+}-ATPase preparation containing mainly the proteins in bands 1, 2, 4.5, 5, and 7. They stated that for enzymatic activity it was essential that spectrin was associated with one or more of these components. Wolf et al. [4] purified a high-affinity Ca^{2+}-ATPase from human erythrocyte membranes by molecular-sieve chromatography in the presence of a nonionic detergent and phosphatidylcholine. The enzyme was assumed to consist of three different subunits with molecular weights of

* To whom correspondence should be addressed.
** Present address: Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, 5 Dong Tan San Tian, Beijing, China.

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145 000, 115 000 and 105 000. In all these preparations of high- or low-affinity Ca^{2+}-ATPase the enzyme has been present in fractions containing other membrane proteins as well. It has therefore been speculated that these enzymes might be active only in complex with other proteins. We present a method for the purification of a low-affinity Ca^{2+}-ATPase which is active in the absence of other proteins. No detergent was used in any purification step. The enzyme is not dependent on magnesium ions for its activity and is accordingly distinct from the high-affinity Ca^{2+}-ATPase.

Materials and Methods

Preparation of cell membranes. Human blood (fresh or recently outdated) was obtained from the blood bank of the University Hospital, Uppsala. The erythrocytes were washed three times by centrifugation in an isotonic buffer (10 mM Tris-HCl buffer, pH 7.5, containing 0.9% NaCl). The washed cells were lysed by the addition of 8 vol. of the 10 mM Tris-HCl buffer (without NaCl) and the membranes were isolated by molecular-sieve chromatography on Sepharose® 4B in the same buffer [5].

Solubilization of membrane proteins. The membranes were extracted with 0.5 mM EDTA and 0.5 mM Tris-HCl buffer (pH 8.0) for at least 24 h. The water-soluble proteins were collected in the supernatant after low temperature centrifugation at 100 000 × *g* for 90 min. The pellet was discarded and the protein solution was stored in the refrigerator.

Agarose suspension electrophoresis [6]. The agarose was prepared by method IIIb in Ref. 7. The 0.16% agarose suspension was made in 0.05 M Tris acetate buffer, pH 8.0. The volume of the sample was 2 ml and its protein concentration 2 mg/ml. The electrophoresis was carried out at 15 mA (800 volts) for 15–17 h. The temperature of the cooling water was about 13°C.

Polyacrylamide gel electrophoresis. Discontinuous electrophoresis was performed in a gel of the dimensions 10 × 10 × 0.15 cm. The stacking and separation gel compositions were $T = 2.5\%$; $C = 2.5\%$ and $T = 6.7\%$; $C = 2.5\%$, respectively (T is the total concentration (in percent) of acrylamide and N,N'-methylenebisacrylamide, expressed as the number of grams of these monomers dissolved in 100 ml of buffer; C is the cross-linking concentration (in percent), i.e. the ratio between the weight of the N,N'-methylenebisacrylamide and the total weight of the monomers, multiplied by 100 [8].) The buffer system was the same as that used by Dewald et al. [9], with the exception that 0.05 M glycine-NaOH, pH 9.8, was employed in the electrode vessels. In some experiments the glycine buffer was used also in the gels (continuous system) and the result was the same as with the discontinuous system. This was expected since the pH (measured) in the gel in the latter system was around 9.8 [10]. Sucrose and tracking dye (Bromothymol blue) were added to the sample (0.1 ml) before it was layered on the gel. Electrophoresis was carried out at 10 mA (80 V) for 6 h. After electrophoresis the gel was cut longitudinally to give 10-cm strips with a width of about 1 cm. One strip was stained with 0.05% Coomassie Brilliant Blue R-250 in 25% (v/v) methanol with 7% (v/v) acetic acid for 1 h and destained with methanol/acetic acid/water (1 : 1 : 8, v/v). By comparison with this stained strip we could easily determine the position of the protein bands in a nonstained strip. After granulation of the gel slice containing the protein band of interest by pressing the gel slice through a net, the protein was extracted with 0.25 M Tris-HCl, pH 8.0 and assayed for ATPase activity. Alternatively the enzyme in a non-stained gel slice (about 2 mm wide) was recovered from the gel by electrophoresis against a sucrose gradient (see below); another method to recover proteins from gel slices is described under Isolelectric focusing (see also Ref. 11) Proteins A and B (Figs. 4 and 8) were recovered by one of these methods. For estimation of the amino acid composition of proteins α and β (Fig. 5) we extracted the Coomassie Brilliant Blue-stained proteins from the gel slices by SDS. The stain was then dissociated from the protein by 8 M urea, which was removed by dialysis against 0.05 M NH_4HCO_3. The solutions of proteins α and β were then freeze-dried and analyzed for the amino acid content.

Discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed in a 10 × 10 × 0.15 cm gel, as described by Neville [12].

For estimation of the molecular weight of the enzyme we used the following standard proteins: phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20000), and α-lactalbumin (14400) from Pharmacia Fine Chemicals, Uppsala, Sweden.

Pore gradient electrophoresis. A Pharmacia gel (PAA 4/30) was prerun for 1 h at a current of 10 mA (70 V) with 0.05 M glycine-NaOH, pH 9.8, in the electrode vessels. The samples, recovered by electrophoresis against a sucrose gradient (see below), were then submitted to pore gradient electrophoresis at room temperature for 17 h at 15 mA (100 V).

Isoelectric focusing. This experiment was performed in a carrier-free medium as described previously [13]; stabilization against convection is achieved by rotation of the horizontal electrophoresis tube around its long axis [14]. The tube was filled with a 1% Ampholine solution (from LKB-Products, Stockholm, Sweden); 0.2 M phosphoric acid and 0.2 M sodium hydroxide were used as anode and cathode solutions, respectively. As samples we used protein-containing gel slices cut from a polyacrylamide gel electrophoresis slab. This recovery technique has the great advantage that the proteins need not be extracted from the gel prior to the application into the electrophoresis tube; the yield of the protein samples is therefore virtually 100%, which is very important when small amounts of material are handled as was the case in these experiments. The focusing was run overnight at a current of 0.3 mA (500 V). The temperature of the cooling water was 10°C. After completion of the run 1-cm fractions were withdrawn from the rotating electrophoresis tubes for pH determinations and enzyme activity measurements.

Concentration of membrane proteins. The enzyme was recovered from 2 mm wide gel slices and at the same time concentrated by electrophoresis against a sucrose gradient containing sodium chloride [15]; 0.05 M glycine-NaOH, pH 9.8, was used as buffer. Ultrafiltration in a collodion bag (from Sartorius, F.R.G.) was used to concentrate the water-soluble protein extract and the ATPase fraction from the agarose suspension electrophoresis.

Assay of Ca^{2+}-ATPase activity. ATPase activities were measured by determining spectrophotometrically inorganic phosphate released from ATP in a total reaction volume of 500 μl [3]. The final concentrations of $CaCl_2$ and ATP were 10 mM and 2 mM, respectively, in 25 mM Tris-HCl buffer, pH 8.0. The samples were incubated at 37°C for 30 min. The reaction was stopped by the addition of 250 μl of cold 20% trichloroacetic acid. The results are expressed in μmol P_i/mg protein per h.

Amino acid analysis. The samples were hydrolyzed for 24 h in 6 M HCl at 110°C. The amino acid content was determined with a Durrum D-500 amino acid analyzer. Total half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation.

Results

Fractionation by agarose suspension electrophoresis

The result of an agarose suspension electrophoresis is shown in fig. 1. The four pools (I-IV) were analyzed for Ca^{2+}-ATPase activity (the histogram in Fig. 1) and for protein content by polyacrylamide gel electrophoresis in SDS (Fig. 2). As shown in Fig. 2, pool I contained very little protein, pool II contained mainly bands 1, 2, 4 and 7, and pool III showed bands 1, 2, 4, 5 and 7,

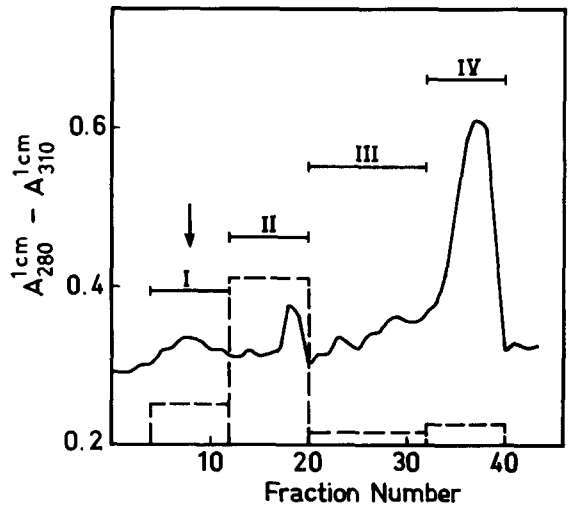

Fig. 1. Agarose suspension electrophoresis. Sample: concentrated water-soluble extract from erythrocyte membranes. The fractions were pooled as indicated. Each of the four pools (I–IV) were analyzed for Ca^{2+}-ATPase activity. The sample was applied at the arrow.

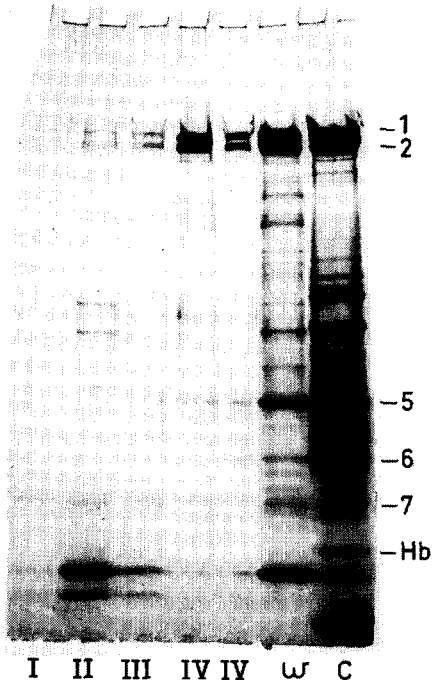

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. I–IV: pools I–IV in Fig. 1. w: water-soluble extract from erythrocyte membranes (= the sample in fig. 1). C: crude membrane. The numbering (1–7) is made according to Steck's nomenclature [16].

pool IV showed bands 1, 2 and 5. The highest activity was found in pool II, which was therefore selected for further purification.

The Ca^{2+}-ATPase activity in the different fractionation steps

The specific Ca^{2+}-ATPase activities (μmol P_i/mg protein per h) of the crude membrane; the water-soluble extract; pool II in Fig. 1; fraction A in Fig. 4 (Fig. 8) were $0.11^{\times}$, $0.53^{\times}$, 1.1^{+}, and 2.9^{+}, respectively ($\times$ and $+$ refer to mean values from five and three identically performed experiments, respectively). The specific activity of the purified protein was accordingly 26-times higher than that of the starting material.

Effect of Mg^{2+} on the ATPase activity.

Fig. 3a shows the Ca^{2+}-ATPase activity at various magnesium ion concentrations. The curve obtained indicates that the Ca^{2+}-ATPase activity was independent of magnesium. Fraction A in Fig. 4

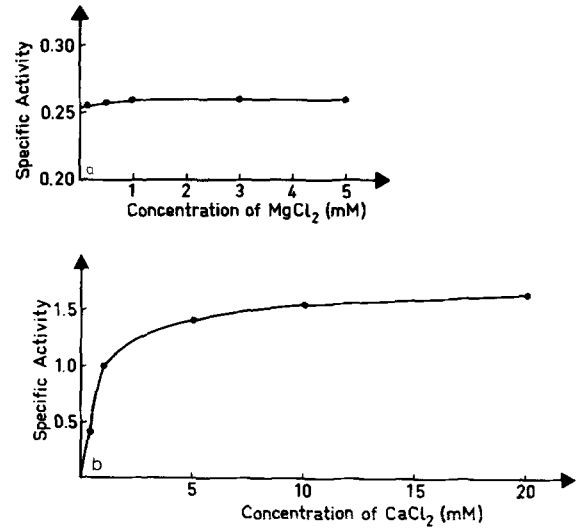

Fig. 3. (a) The Ca^{2+}-ATPase activity as a function of the concentration of Mg^{2+}. Sample: Fraction A in Fig. 4. (b) The Ca^{2+}-ATPase activity as a function of the concentration of Ca^{2+}. Sample: Fraction A in Fig. 4.

was used for these measurements, as well as those described in the next two paragraphs. The amount of magnesium in fractions A and B (in Fig. 4) was determined by atomic absorption photometry to be 0.009 and 0.008 μmol Mg^{2+}/mg protein. The endogeneous content of magnesium is accordingly far less than that required for activation of a (Ca^{2+} + Mg^{2+})-ATPase [17].

Effect of Ca^{2+} on the ATPase activity.

Fig. 3b shows that for optimal activity Ca^{2+} concentrations above 10–20 mM are required (curves very similar to that shown in Fig. 3b were obtained in two other experiments).

Effect of ATP on the ATPase activity

From a Lineweaver-Burk plot (the reciprocal of activity plotted against the reciprocal of the ATP concentration) the K_m value for ATP was determined to be 63 μM.

Amino acid analysis

Table I shows the amino acid compositions of fractions A and B in Fig. 4 (or Fig. 8) and fractions α and β in Fig. 5. Interestingly, the amino acid compositions of all four fractions exhibit many

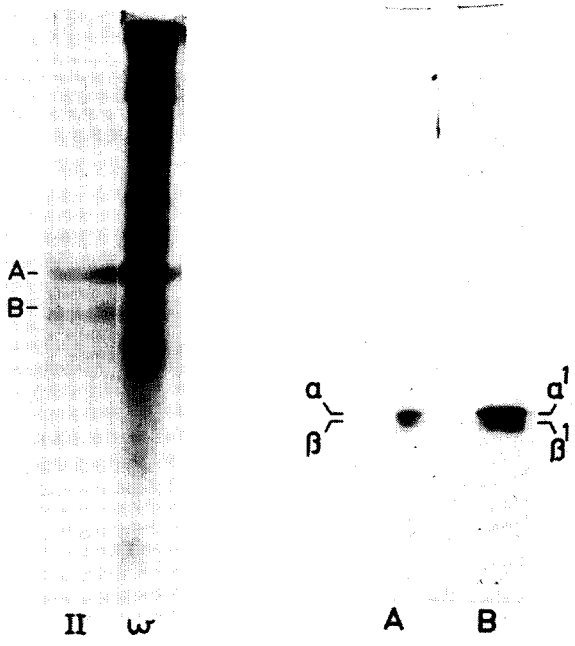

Fig. 4. Polyacrylamide gel electrophoresis (in the absence of detergents). II: fraction II from agarose suspension electrophoresis in Fig. 1. w: water-soluble extract from erythrocyte membranes. Another electrophoretic analysis of pool II is shown in Fig. 8.

Fig. 5. Polyacrylamide gel electropgoresis in sodium dodecyl sulfate. A and B: fractions A and B, respectively, from polyacrylamide gel electrophoresis in Fig. 4.

similarities. The content of tyrosine could not be determined for fraction A since the sample of this fraction had been oxidized with performic acid and the HBr used to destroy the excess oxidant leads to destruction of tyrosine. The samples of fractions B, α and β were hydrolyzed without prior oxidation, so half-cystine could not be determined accurately. The quantity of glycine is not shown in the table, because glycine buffer was used in the electrophoresis. The quantity of arginine in fractions α and β were not determined because urea was not completely removed in the dialysis step, and the large amount of ammonia produced during hydrolysis necessitated termination of the run before elution of ammonia.

Since the protein quantities in fractions A, B, α and β were very low several gel electrophoresis experiments had to be done to get material enough

Table I

THE AMINO ACID COMPOSITION OF PROTEINS A AND B IN Fig. 4; AND α AND β IN Fig. 5

For all four proteins the concentration of threonine has arbitrarily been set eqal to 1; n.d., not determined.

	A	B	α	β
Asp	2.30	2.35	1.77	1.61
Thr	1.00	1.00	1.00	1.00
Ser	1.80	1.54	2.04	1.33
Glu	2.95	2.56	3.03	2.95
Pro	0.69	0.96	0.64	0.66
Ala	1.53	1.53	1.60	1.65
Val	0.93	1.34	1.00	1.03
Met	0.19	0.07	0.14	0.10
Ile	0.82	0.75	0.87	0.85
Leu	1.61	1.52	1.72	1.82
Tyr	n.d.	0.55	0.58	0.47
Phe	0.54	0.76	0.54	0.55
His	0.29	0.25	0.19	0.12
Lys	0.96	0.96	1.02	1.13
Arg	0.79	0.80	n.d.	n.d.
Cys	0.33	n.d.	n.d.	n.d.

for an amino acid analysis. Therefore and also because there were many steps involved in the recovery of the proteins following gel electrophoresis, particularly for fractions α and β, the accuracy in the amino acid analyses is not high.

Homogeneity of the enzyme.

Fig. 4 shows the pattern obtained with fraction II from the agarose suspension electrophoresis in Fig. 1 upon polyacrylamide gel electrophoresis. Fig. 5 shows an analysis of the fractions A and B from the polyacrylamide gel electrophoresis in Fig. 4 (Fig. 8) by polyacrylamide electrophoresis in SDS. The two bands obtained (α, β, and α^1, β^1, respectively) are located very close to each other and therefore correspond to proteins of similar molecular weights (23 000; 21 000). Upon pore-gradient electrophoresis in the absence of detergents fractions A and B gave identical patterns; one band (or possibly two close bands) corresponding to a molecular weight of about 28 000 (Fig. 6). In isoelectric focusing both A and B gave two enzymatically active zones with pH-values around 4.9 and 3.9 (Fig. 7). The amino-acid compositions of fractions A and B were similar and

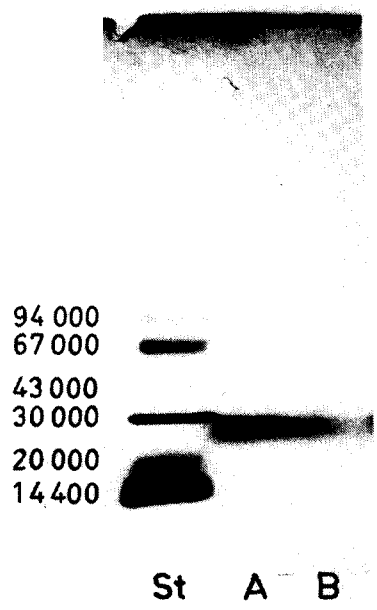

Fig. 6. Pore gradient electrophoresis of the fractions A and B from the experiment shown in Fig. 4. St: standard proteins.

similar to those of α and β (Table I).

The ratio between the amounts of protein in bands α and β (Fig. 5) was 2, 4, 4.5, 9 (four experiments) and in bands α^1 and β^1 2.4, 2.5 (two experments), as estimated by scanning the stained gels at 470 nm.

We have not found any alkaline phosphatase activity in fractions A and B (in Fig. 4) against the following substrates: uridine 5′-triphosphate, cytidine 5′-triphosphate, β-glycerol phosphate, and glucose 6-phosphate.

Discussion

The risk of misinterpretation of experimental data due to the tendency of membrane proteins to aggregate

One of the most common methods to purify proteins is to precipitate them by polymers, such as polyethylene glycol, dextran, methyl cellulose. One can therefore expect that proteins coming into contact with gels made up of these and other polymers also will exhibit a decreased solubility (although the decrease is less pronounced than with the free polymer molecules). This tendency to precipitate (or form aggregates) is, of course, particularly great for proteins with poor water solubility, such as the ATPase studied (and many other membrane proteins). This may explain the observation we have often made that membrane pro-

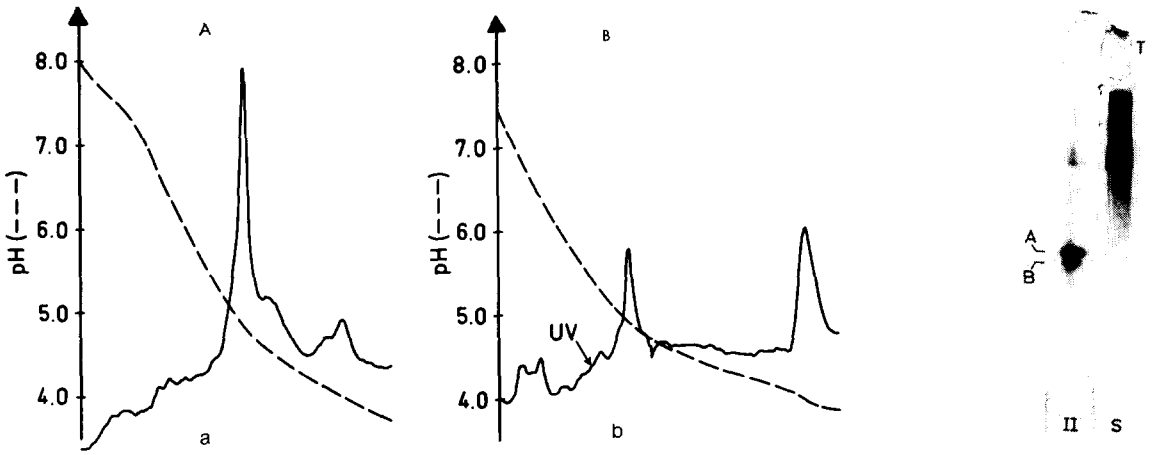

Fig. 7. Isoelectric focusing. (a) Sample: fraction A in Fig. 4. (b) Sample: fraction B in Fig. 4. The full curve corresponds to a scan of the tube at 280 nm after electrophoresis for 17 h. The minor peaks refer to ultraviolet light absorbing impurities in the Ampholine solution. Ca^{2+}-ATPase activity was found in the zones with the pH values around 4.9 and 3.9.

Fig. 8. Polyacrylamide gel electrophoresis (in the absence of detergents). II: Pool II from an agarose suspension electrophoresis, similar to that shown in Fig. 1. S: enzymatically active fraction from a chromatographic experiment on Sepharose 4B (not shown here). The ATPase activity was found in bands A and B for sample II and at the top T of the gel for sample S, indicating that the Sepharose bed caused aggregation of proteins.

teins (also in the presence of detergents) precipitate or form large aggregates upon chromatography or electrophoresis in supporting media (for a more thorough discussion, see Ref. 18). For example, when the water-soluble protein extract was chromatographed on Sepharose 4B and the fraction with ATPase activity were analyzed by polyacrylamide gel electrophoresis the enzyme activity was found at the top T of the gel (Fig. 8, sample S). Evidently, the Sepharose column caused the enzyme molecules to aggregate with themselves or with other (inactive) proteins. However, when the same protein extract was purified by agarose suspension electrophoresis (instead of Sepharose chromatography), the enzymatically active fractions gave two well-defined bands with ATPase activity upon polyacrylamide gel electrophoresis (A and B in Fig. 8, sample II). The agarose concentration in the chromatographic experiment was about 23 ($= 4.0/0.17$) times higher than in the electrophoresis run, which may explain the greater tendency to form aggregate in the former experiment.

All of the authors mentioned in the Introduction have used molecular-sieving on agarose for the purification of ATPase and have reported that the enzyme was found in fractions containing other proteins. Could this finding be explained by these aggregation phenomena? In any case, by avoiding the gel chromatographic step we have isolated two active forms of a low affinity Ca^{2+}-ATPase (A and B in Fig. 4 or Fig. 8), which do not show association with other membrane proteins to judge from a polyacrylamide gel electrophoresis in SDS (Fig. 5) and from a pore-gradient electrophoresis in the absence of detergents (Fig. 6),

The purified Ca^{2+}-ATPase requires the presence of Ca^{2+} (but not of Mg^{2+}) and in this respect resembles the enzyme purified by White and Ralston [3]. However, their enzyme preparation had a 26-fold lower specific activity, indicating a lower degree of purification. Because the ATPase isolated does not require Mg^{2+} (Fig. 3a) but needs high concentrations of Ca^{2+} (approx. 10 mM) (Fig. 3b) for activity it should be classified as a low affinity Ca^{2+}-ATPase.

A comparison between the components A and B.

A and B gave the same pattern in SDS electrophoresis, i.e. the two bands α, β and α^1, β^1, respectively, in Fig. 5, corresponding to the molecular weights 23 000 and 21 000. However, the ratio between the amounts of protein in bands α and β is in general not the same as that in bands α^1 and β^1; these ratios can also vary considerably from experiment to experiment. In pore-gradient electrophoresis (in the absence of detergents) forms A and B also gave identical patterns: one band (or possibly two very closely situated bands) corresponding to a molecular weight of about 28 000. In carrier-free isoelectric focusing form A gave two enzymatically active zones, a major one at pH 4.9 and a smaller one at pH 3.9 (Fig. 7a); form B gave a major peak at pH 3.9 and a somewhat smaller peak at pH 4.9 (Fig. 7b). As shown in Table I the amino acid compositions of α and β were similar and similar to those of A and B (as mentioned above the amino acid analyses are not sufficiently accurate to permit more detailed conclusions).

These observations prompt the following hypotheses and question:

I. Components α and β are probably identical to components α^1 and β^1, respectively (Fig. 5).

II. Are fractions A and B mixtures of components α and β; and α^1 and β^1, respectively, (which cannot be resolved by poly-acrylamide gel electrophoresis in the absence of SDS; Fig. 4 or Fig. 8)?

If conclusion I is correct, i.e. if component α is identical to component α^1 and component β is identical to component β^1 a mixture of α and β (= fraction A) should give the same electrophoresis pattern as a mixture of α^1 and β^1 (= fraction B) upon polyacrylamide gel electrophoresis not only in the presence but also in the absence of SDS. As shown in (Fig. 4 and Fig. 8) this is not the case and therefore alternative II is not likely.

III. Fractions A and B are both complexes of components α and β (we now assume that $\alpha = \alpha^1$ and $\beta = \beta^1$, i.e. that conclusion I is correct). The difference between the complexes A and B can then be related to the difference in the number of molecules of α and β constituting the complexes. Since the ratio between the amounts of α and β varies from experiment to experiment one can conclude that neither A nor B has a fixed stoichiometric composition of the components α and β. On the other hand the migration distances for fractions A and B have been reproducible in all

experiments, which indicates that the molecular size of A does not vary very much, nor does that of B. This means that the sum of the number of the α and β subunits constituting A is relative constant; the same is true for B, although for this molecule the sum should be smaller since it migrates faster than A in the polyacrylamide gel electrophoresis (Fig. 4 or Fig. 8). This result is not surprising, since polymers consisting of identical or similar subunits can be expected to form stable configurations only when the number of subunits has a certain fixed value. Whether the native form of Ca^{2+}-ATPase is a polymer or consists of one α or one β unit is an open question. The result that only one band (or possibly two close bands) corresponding to a molecular weight around 28 000 was obtained in pore-gradient electrophoresis in the absence of detergent (Fig. 6) need not favour the latter alternative, but may indicate that the polymers A and B are not quite stable but can dissociate into the monomers also in a detergent-free milieu. This dissociation is of course much more rapid in pore-gradient electrophoresis than in conventional polyacrylamide gel electrophoresis.

Rosenthal and Kregenow [19] extracted water-soluble proteins and studied the ATPase activity in a spectrin fraction. They found that the enzyme was inhibited by magnesium ions. Kirkpatrick et al. [20] and Weidekamm and Brdiczka [21] also reported a Ca^{2+}-ATPase in a spectrin-actin fraction. Avissar et al. [2] and White and Ralston [3] pointed out that the spectrin did not have Ca^{2+}-ATPase activity of its own, but only in association with one or more other proteins. The purified Ca^{2+}-ATPase we have studied is a protein which does not form complexes with actin (band 5) or other proteins which can be resolved by SDS electrophoresis. The enzyme is a band 7 protein. We have not attempted to localize the enzyme in the membrane, and therefore we cannot exclude the possibility that it is, for instance, a cytoskeletal protein [22].

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