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LIMITED PROTEOLYSIS OF HUMAN ERYTHROCYTE Ca^{2+}-ATPase IN MEMBRANE-BOUND FORM. IDENTIFICATION OF CALMODULIN-BINDING FRAGMENTS

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SUMMARY: Water-soluble and membrane-bound calmodulin-binding polypeptides formed upon limited proteolysis of erythrocyte ghosts were isolated by means of affinity chromatography. Immune blotting revealed that all isolated fragments originated from Ca^{2+}-ATPase. Among the fragments obtained those having formed an acylphosphate intermediate were identified. The N-terminal residue of purified intact Ca^{2+}-ATPase was shown to be blocked (probably acylated).

Proteolytic enzymes procure essential information on the molecular organization of membrane proteins. Limited proteolysis of erythrocyte Ca^{2+}-ATPase was thoroughly studied on the purified solubilized enzyme (1,2,3). Since a solubilization procedure may cause considerable conformational rearrangements of a protein molecule, investigations of the membrane-bound enzyme would be a valuable independent approach to the problem. However, isolation of homogeneous membrane-bound preparation of Ca^{2+}-ATPase was not described, therefore we subjected erythrocyte ghosts to a limited trypsin treatment. As a result, the Ca^{2+}-ATPase polypeptide fragments interacting with calmodulin were isolated by affinity chromatography and characterized by SDS-gel electrophoresis.

MATERIALS AND METHODS

All reagents were of the highest purity available. Trypsin was obtained from Worthington, soybean trypsin inhibitor was from Calbiochem, L-α-phosphatidylcholine (type III S) from Sigma, (γ-^{32}P)-ATP was from Amersham. Erythrocyte membranes were prepared by a combination of two methods (4,5). The

Abbreviations: EDTA, ethylene diamine N,N,N',N'-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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solubilization of membrane proteins in Triton X-100 and purification of Ca^{2+}-ATPase on a calmodulin affinity column were performed as in (4).

ATPase activity was determined at 37°C for 10 min in the medium containing 130 mM KCl, 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.2 mM ouabain, 0.5 mM ATP, 0.05 mM $CaCl_2$ (6). Protein concentrations were determined as in (7). Amino acid analyses of Ca^{2+}-ATPase were carried out on a Durrum autoanalyzer D-500. Cystein and methionine were determined according to (8), tryptophan - as in (9). N-terminal amino acid residue was investigated by dansylation of the samples in the presence of SDS, by the pyroglutamic acid cycle opening (10) and by deformylation (11).

Limited tryptic digestion of Ca^{2+}-ATPase in erythrocyte membranes (2 mg of the protein/ml) was carried out in buffer A (130 mM KCl, 20 mM HEPES, pH 7.4, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$) with 0.04 mg of trypsin/ml at 4°C for 2 min. The reaction was stopped by addition of 3-fold excess (w/w) of soybean trypsin inhibitor. The final hydrolyzate was then centrifuged at 100,000·g for 60 min at 4°C. Supernatant (fraction I) was applied to a Sepharose 4B calmodulin column without any previous treatment. The resin was equilibrated in buffer A. Calmodulin fragments were eluted with buffer B (same composition as buffer A, except that 0.05 mM $CaCl_2$ was replaced by 4 mM EDTA). The pellet of trypsin-treated membranes (fraction II) was washed twice in buffer A and calmodulin-binding fragments were isolated by a procedure of (4).

Phosphorylation of Ca^{2+}-ATPase fragments was carried out in buffer A (for fraction I) or in buffer A containing 0.1% Triton X-100, 0.05% phosphatidylcholine (for fraction II) at 0°C according to (12). Hydroxylamine treatment was done as in (13). SDS-polyacrylamide gel-electrophoreses of Ca^{2+}-ATPase (7% polyacrylamide) and of tryptic fragments (10% polyacrylamide) were performed by method (14). The radioactive gels were stained for proteins, dried, and exposed to RMV X-Ray film (Tasma, USSR) using intensifying screens for 3 days at -70°C. Production of antiserum and purification of immune IgG were performed as in (15). Immune blotting of Ca^{2+}-ATPase tryptic fragments was performed by SDS-polyacrylamide gel-electrophoresis followed by capillary transfer to nitrocellulose sheets. The procedure of staining the nitrocellulose blot was as in (16), except that peroxidase-labelled goat anti-(rabbit IgG)antibodies and 4-chloro-1-naphtol were used.

RESULTS AND DISCUSSION

To obtain the erythrocyte membranes in the amounts sufficient for structural studies of Ca^{2+}-ATPase a Pellicon Cassette System was applied. As a result, the yield of the membranes from 2 litres of packed erythrocytes was increased up to 10 g (Ca^{2+}-ATPase sp. activity - 0.016 $\mu mol \cdot min^{-1} \cdot mg^{-1}$; in the presence of calmodulin - 0.06 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). Electrophoretically homogeneous Ca^{2+}-ATPase displayed sp. activity of 0.35 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and was 7-10 fold activated by calmodulin.

The obtained samples were used for the preliminary chemical characterization of the enzyme and in production of antiserum. The amino acid composition was determined to be (based on a molecular weight of 140,000, mol/mol): Asx 124; Thr 79; Ser 103; Glx 122; Pro 71; Gly 186; Ala 134; Cys 30; Val 109; Met 23; Ile 80; Leu 125; Tyr 21; Phe 41; His 19; Lys 67; Arg 37; Trp 6.

These estimates deviate considerably in the quantities of some amino acids (e.g. Ser, Gly, Cys, Lys, Arg, Ala) from those reported earlier and should correspond to a more hydrophobic molecule than that calculated on the basis of the data shown in (17). Since the N-terminal amino acid residue of the protein was not identified by dansylation even in the presence of SDS as well as after pyroglutamic acid cycle opening and after deformylation, it was assumed to be acylated.

Conditions for proteolytic treatment of erythrocyte membranes were selected taking into account two main criteria: a) Ca^{2+}-ATPase or its fragments in the final mixture should retain ATPase activity as well as the ability to be activated by calmodulin; b) the resultant fragments should not be too numerous.

As shown in Fig. 1, at trypsin concentrations lower than 60 μg/ml membrane bound Ca^{2+}-ATPase exhibits the increasing trypsin-stimulated activity while retaining the ability for calmodulin activation. At higher trypsin concentrations the calmodulin sensitivity is lost. (The analogous curve of trypsin activation was demonstrated for the purified enzyme reconstituted into phospholipid vesicles (1)). Based on the above results and the data of

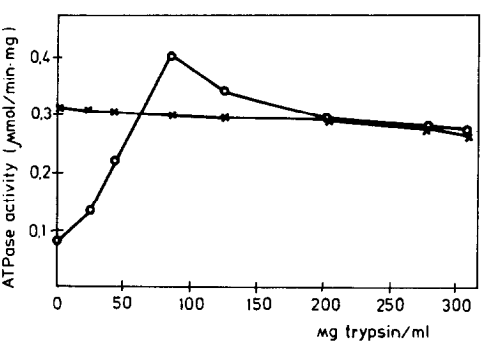

Fig. 1. Activation of the Ca^{2+}-ATPase in calmodulin-deficient membranes by limited proteolysis. Ca^{2+}-ATPase of disrupted erythrocyte membranes (protein concentration 2 mg/ml) was digested by varying amounts of trypsin at 4°C. The reaction was terminated after 2 min by addition of a 3-fold excess (w/w) of soybean trypsin inhibitor, and the ATPase activity was assayed immediately, in the absence and in the presence of calmodulin. Calmodulin (1 μg/10 μg of the protein) was introduced prior to ATP addition. For the control (0 μg of trypsin in the figure) membranes were incubated with 60 μg of inhibitor and 20 μg of trypsin added together for 2 min at 37°C.

electrophoretic analyses (not shown) the following conditions were selected: trypsin-protein ration = 1:50, w/w; 2 min at 4^0C. After proteolysis and separation of the hydrolyzate into water-soluble (I) and membrane-bound (II) fractions, the former was directly applied to calmodulin-Sepharose column. Isolation of the fragments in the absence of detergent prevents their possible denaturation. Fraction II was treated with 0.5% Triton X-100 before loading onto the column. In both cases after the extensive wash of the column with Ca^{2+} buffers the calmodulin-binding fragments were eluted with EDTA-buffers (Fig. 2, lines A_1, B_1). Fraction I contains three apparent polypeptide bands of Mr=28,000, 53,000 and 82,000, several polypeptides of higher Mr and minor products. The most evident degradation products of fraction II are polypeptides of Mr=28,000, 33,500, 53,000. A comparison of the patterns shows that 28,000 and 53,000 Mr fragments are common, while other polypeptides are specific for each fraction.

The SDS-gels were blotted onto nitrocellulose sheets and tested with antibodies raised against purified Ca^{2+}-ATPase. Fig. 2 (lines A_3, B_3) indicates that all of the bands revealed in the Coomassie stained gels (A_1, B_1) correspond to Ca^{2+}-ATPase proteolytic degradation products.

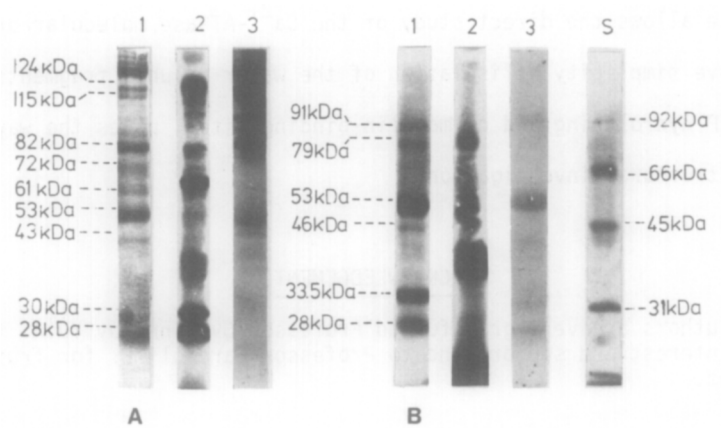

Fig. 2. Gel-electrophoretic analyses of water-soluble (A) and membrane-bound (B) calmodulin-binding fragments of Ca^{2+}-ATPase. 1) Coomassie blue-stained gel. 2) Autoradiogram of the (γ-^{32}P)-ATP treated fragments. 3) Immune blotting of the gel shown in 1. S. Molecular weight standarts: phosphorylase b (92.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa).

Thus four main fragments were found to possess calmodulin-binding activity while in the case of solubilized Ca^{2+}-ATPase this function was assigned only to two polypeptides (5).

Both EDTA-eluates possessed ATPase calmodulin-dependent activity. To identify the fragments containing the active site of Ca^{2+}-ATPase the EDTA-eluate samples were treated with (γ-^{32}P)-ATP (Fig. 2, lines A_2, B_2). Polypeptides of Mr=28,000, 30,000, 37,000-40,000, 61,000-72,000, 85,000 and some of the higher Mr fragments of fraction I as well as the fragments of Mr=37,000-40,000, 53,000, 79,000 of fraction II become phosphorylated. The radioactively labelled polypeptides were proved to be acylphosphates since they were sensitive to hydroxylamine treatment.

Noteworthy are some other properties discovered:

1) poorly stained fragments of Mr=37,000-40,000 are intensely phosphorylated;

2) The fragment of Mr=33,500 was found only in Fraction II and was not phosphorylated. Evidently, it forms the membrane portion of the protein molecule and contains only the calmodulin-binding site. So, it could be suggested that this site is located closer to the membrane segment of the polypeptide chain than the ATP-hydrolyzing site.

The proposed approach to the proteolytic treatment of membrane-bound Ca^{2+}-ATPase allows the direct study of the Ca^{2+}-ATPase molecular organization. The relative simplicity of isolation of the water-soluble fragments containing ATP-hydrolyzing and calmodulin-binding sites, paves the way to their thorough structural investigation.

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