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Conformational changes of the in situ red cell membrane calcium pump affect its proteolysis

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In inside-out red cell membrane vesicles trypsin digestion reduces the molecular mass of the ^{32}P-labeled acyl-phosphate intermediate of the calcium pump from the original 140 kDa to about 80 kDa with a simultaneous activation of the calcium uptake. This process is slightly stimulated by the presence of calcium, as compared to EGTA, or EGTA + vanadate, but the proteolytic pattern is similar under all these conditions. However, trypsin degradation of the 80 kDa polypeptide, resulting in the loss of calcium transport activity and ^{32}P-phosphoenzyme formation, is rapid in the presence of calcium, inhibited by EGTA and almost fully blocked by EGTA + vanadate. In the presence of these latter ligands, probably locking the calcium pump in an E_2 conformation, the 80 kDa protein becomes insensitive even to excessive digestion by the non-specific protease, pronase. The data indicate major changes in the molecular arrangement of the calcium pump protein when transformed from a calcium-liganded (E_1) to an E_2 conformation.

Controlled proteolytic digestion of the red cell membrane calcium pump proved to be a useful method for connecting structural and functional characteristics of this transport system (see Refs. 1–8). Studies on the in situ [1,2,4] or on the isolated enzyme [3,5–8] showed that partial proteolysis of the pump protein induced a calmodulin-like activation while eliminated the action and binding of calmodulin. The proteolytic degradation products and the effects of various ligands of the pump on its proteolysis were carefully analyzed by using purified erythrocyte calcium pump preparation [7,8]. Recently we have developed a sensitive method for the analysis of the in situ calcium pump in inside-out red cell membrane vesicles, where the calcium pump represents only about 0.2% of the total membrane proteins. The method is based on the formation of a maximum level ^{32}P-phosphoenzyme from [γ-^{32}P]ATP in the presence of lanthanum and the analysis of the phosphoproteins in a special, acidic SDS-PAGE slab [9]. Calcium transport and its calmodulin-sensitivity were simultaneously measured in the proteolyzed inside-out vesicle preparations. By using this method we found that the in situ pump had a similar trypsin degradation pattern to that ob served in the isolated protein: intermediary fragments of 125, 90 and 80 kDa could be observed. The two former fragments retained calmodulin sensitivity, while the 80 kDz fragment was a fully active, calmodulin-insensitive calcium transporter [9]. Further proteolysis eliminated both active calcium uptake and the formation of the ^{32}P-phosphoenzyme. In contrast to the findings with the isolated calcium pump [7] in the inside-out vesicle

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membranes there was no smaller ^{32}P-phosphoenzyme-forming fragment than the 80 kDa polypeptide [9].

In the present study, stimulated by the work of Jørgensen [10] related to the conformational changes of the Na^+-K^+ pump, we attempted to follow the proteolytic degradation of the in situ calcium pump in inside-out vesicles by locking the enzyme in two different basic conformations. The calcium-liganded form of the pump protein represents its E_1 state. In contrast to this, the chelation of calcium ions by EGTA and especially the combination of EGTA and vanadate (a high-affinity reversible inhibitor of the calcium pump, see Refs. 11–13) convert the enzyme into an E_2 state [6,14,15].

In the following experiments limited proteolysis of the inside-out vesicle membranes was carried out in the presence of various combinations of calcium, EGTA and vanadate. The vanadate concentration applied (50 μM) fully blocked active calcium uptake but the inhibition was completely reversible upon washing of the membranes. After stopping the proteolysis the vesicles were washed free of the above ligands and tested for active calcium uptake. Identical samples were phosphorylated by [γ-^{32}P]ATP in the presence of calcium + lanthanum. Under the phosphorylation conditions applied (no magnesium, 60 s phosphorylation at 4°C) a maximum level of the calcium pump ^{32}P-phosphoenzyme was formed without the labeling of any other proteins. The phosphoproteins were analyzed in a discontinuous acidic SDS-PAGE slab system followed by the autoradiography of the dried gels [9].

The results of mild trypsin exposure of the inside-out vesicle membranes are shown in Fig. 1. During proteolysis the successive formation of the 90 and 80 kDa ^{32}P-phosphoenzyme-forming fragments can be observed and the appearance of this latter fragment coincides with the calmodulin-independent activation of calcium uptake. The proteolytic and the calmodulin stimulation of the calcium pump are not additive but reach the same maximum transport rate (see also Refs. 1–8). As seen in Fig. 1C, there is no major difference in the appearance of the proteolytic products of the ^{32}P-phosphoenzyme-forming pump protein, whether the digestion is carried out in the presence of calcium or EGTA + vanadate. A slight acceleration of the pump-activation and the formation of the 80 kDa fragment is observed in the presence of calcium. These findings are in contrast to the data reported by Rossi and Schatzmann [16], who found an absolute calcium requirement for the trypsin activation of the red cell calcium pump. The origin of this difference is unknown at present.

The major difference as revealed in Fig. 1, is between the calcium pump activity and ^{32}P-phosphoenzyme formation in samples digested for 60 min either in the presence of calcium or EGTA + vanadate. If calcium is present during digestion the transport activity decreases to about one-fifth of the maximum value and practically no ^{32}P-phosphoenzyme is seen on the autoradiogram. Both calcium transport and ^{32}P-phosphoenzyme phosphorylation are conserved if the 60 min trypsin digestion is carried out in the presence of EGTA + vanadate (EGTA itself has a similar, but less effective protecting effect).

Addition of $MgCl_2$ (1 mM) which has been suggested to support the effect of vanadate [13,15] had no significant effect on the trypsin digestion of the inside-out vesicle calcium pump (data not shown). As we plan to report it elsewhere, under certain conditions the 80 kDa band can be further resolved to 81 kDa and 78 kDa fragments, the latter being the phosphorylated limit polypeptide of the calcium pump. The gel system applied in the present study does not allow to distinguish between these fractions.

The protein-stained gels (panel B) clearly show that degradation of most membrane proteins, such as spectrin or Band 3, is not affected by the above ligands.

Fig. 2 shows the effect of pronase digestion on the rate of active calcium uptake by inside-out vesicles. In these experiments again either calcium, calcium + vanadate, EGTA or EGTA + vanadate are added to the membranes during digestion and then the ligands and the proteolytic enzyme are washed off from the inside-out vesicles. A rapid pronase activation of calcium uptake, reaching the level of calmodulin-stimulation, is observed under all conditions. However, in the presence of calcium (with or without vanadate) proteolytic inactivation is rapid, while EGTA, and especially EGTA +

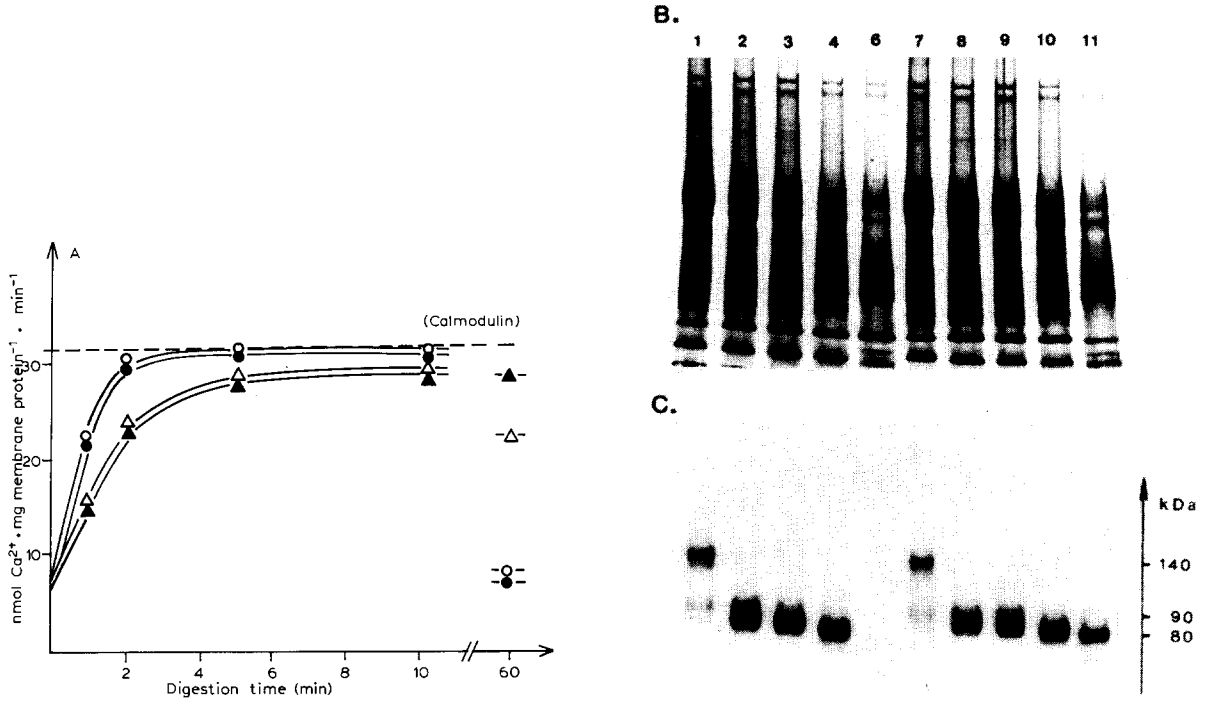

Fig. 1. Effect of mild trypsin digestion of inside-out red cell membrane vesicles (IOVs) on the calcium pump. (A) The rate of calcium uptake (nmol Ca·mg IOV protein^{-1}·min^{-1}) as a function of the time of trypsin digestion. (B) The protein-stained gel after SDS-PAGE separation of the inside-out vesicle proteins. (C) Autoradiogram of the above gel. Trypsin digestion of the inside-out vesicles was carried out at 37° C in a medium containing 100 mM KCl, 20 mM Hepes (pH 7.0), 50 μg/ml trypsin (Sigma), ±100 μM EGTA and ±50 μM Na_3VO_4. Digestion was stopped by 10-times excess of soybean trypsin inhibitor and the vesicles were washed twice in a 100 mM KCl, 20 mM Hepes-K (pH 7.0) solution with centrifugations of 5 min, 10000×g at 4° C. Aliquots of the pretreated membranes were immediately used in the calcium uptake and phosphorylation experiments, respectively. Active calcium uptake by inside-out vesicles was measured by a rapid filtration method with Sartorius membrane filters, as described in Ref. 1. The vesicles were incubated in media containing 100 mM KCl, 20 mM Hepes-K (pH 7.0), 1 mM $MgCl_2$, 0.5 mM ATP and 50 μM $CaCl_2$, including ^{45}Ca tracer, ±0.5 μM calmodulin. Radioactive calcium accumulation was measured by liquid scintillation [1] and the rates were calculated from linear uptake periods of 5 min. Inside-out vesicle membrane phosphorylation and the analysis of ^{32}P-phosphoenzyme were carried out as described in Ref. 9. In brief, phosphorylation was performed by [γ-^{32}P]ATP (0.2 μM) at 4° C for 60 s in a medium contain 100 mM KCl, 20 mM Hepes-K and 50 μM $LaCl_3$. The trichloroacetic acid-precipitated proteins were washed free of unbound ^{32}P activity, the membranes dissolved in an electrophoresis sample buffer and applied to a 5–10% acrylamide gradient slab gel. The stacking part of the gel (4% acrylamide) had a pH of 5.5 adjusted with Tris-phosphate, the pH of the running gel, containing a Tris-phosphate buffer was 6.5. The electrophoresis buffer had a pH of 6.3 and contained Mops as a buffer. Electrophoresis was carried out at 4° C for 3 h. The slabs were stained in Coomassie blue, differentiated in acetic acid and dried. The dried slabs were autoradiographed for 24–72 h. Ligands during trypsin digestion: 20 μM $CaCl_2$: panel A, symbol ○, and panels B and C, lines 1–6; 20 μM $CaCl_2$ + 50 μM Na_3VO_4: panel A, symbol ●; 100 μM EGTA: panel A, symbol △; 100 μM EGTA + 50 μM Na_3VO_4: panel A, symbol ▲ and panels B and C, lines 7–11. Digestion time: lines 1 and 7, 0 min; lines 2 and 8, 2 min, lines 3 and 9, 5 min, lines 4 and 10, 10 min, lines 6 and 11, 60 min.

vanadate protect the calcium pump against this proteolytic inactivation.

In Fig. 3 the formation of the calcium pump ^{32}P-phosphoenzyme after pronase treatment (lines 1–4) is demonstrated. If digestion is carried out for 60 min in the presence of calcium (line 3) no ^{32}P-phosphoenzyme formation is seen. EGTA (line 2) or EGTA + vanadate (line 4) effectively protect the calcium pump ^{32}P-phosphoenzyme during similar time of pronase digestion. As shown in Panel A of Fig. 3, pronase digestion of spectrin or Band 3 is similar under all these conditions. For the

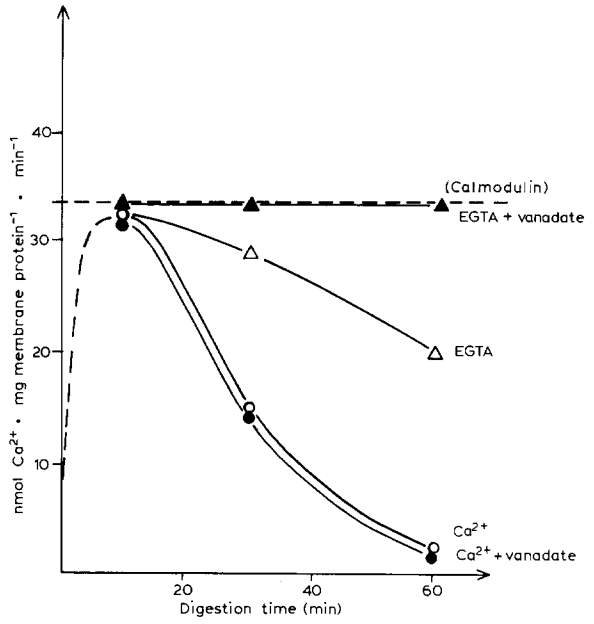

Fig. 2. Effect of pronase digestion of inside-out vesicles (IOVs) on the calcium transport activity. The rate of active calcium uptake (nmol Ca^{2+}·mg IOV protein^{-1}·min^{-1}) is shown as a function of the time of pronase digestion. Pronase was applied at 4° C in 100 μg/ml final concentration in similar media as described in the legends to Fig. 1. Digestion was stopped by the dilution and washing of the inside-out vesicles and calcium uptake was measured by rapid filtration, as described at Fig. 1. The samples contained the following additions during digestion: ○, 20 μM $CaCl_2$; ●, 20 μM $CaCl_2$ + 50 μM Na_3VO_4; △, 100 μM EGTA: ▲, 100 μM EGTA + 50 μM Na_3VO_4.

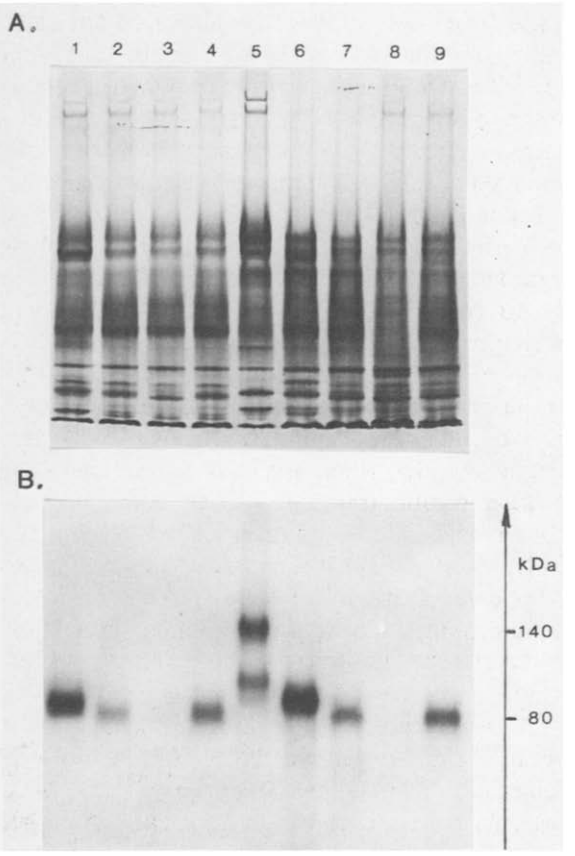

Fig. 3. Effects of pronase and trypsin digestion on the formation of the ^{32}P-phosphoenzyme of the calcium pump in inside-out vesicles. (A) Protein staining of the SDS-PAGE slab; (B) Autoradiography of the same gel. Proteolytic digestion, membrane phosphorylation, SDS-PAGE and autoradiography were carried out as described in the legends to Figs. 1 and 2. Pronase was used in 100 μg/ml final concentration at 4° C; trypsin was used in 50 μg/ml final concentration at 37° C. Line 1, 5 min digestion with pronase, 50 μM $CaCl_2$; 2, 60 min digestion with pronase, 100 μM EGTA; 3, 60 min digestion with pronase, 50 μM $CaCl_2$; line 4, 60 min digestion with pronase; 100 μM EGTA + 50 μM Na_3VO_4; 5, Control (incubated for 60 min at 37° C without added protease); 6, 5 min digestion with trypsin, 50 μM $CaCl_2$; 7, 60 min digestion with trypsin, 100 μM EGTA; 8, 60 min digestion with trypsin, 50 μM $CaCl_2$; 9, 60 min digestion with trypsin, 100 μM EGTA + 50 μM Na_3VO_4.

sake of comparison, Fig. 3 also includes the samples digested with pronase for 5 min (representing the maximally activated pump) and trypsin-digested samples under similar conditions. The control inside-out vesicle preparation has the original 140 kDa calcium pump ^{32}P-phosphoenzyme and some 90 kDa fragments due to endogenous proteolysis during 60 min in this sample.

The above experimental data indicate that certain parts of the red cell membrane calcium pump protein are easily accessible for proteolysis at the cytoplasmic membrane surface. The cleavage of these parts, which do not contribute to the calcium translocation process, only slightly depends on the actual conformation of the pump. In contrast to this, the basic pump unit of the transport protein (the calmodulin-insensitive, maximum activity 80 kDa peptide) significantly changes its molecular conformation when locked in an E_1 or E_2 conformation, respectively. The peptide in its E_2 state becomes practically inaccessible to any proteolytic attack, even if the non-specific enzyme mixture, pronase is applied to the inside-out vesicle mem-

brane. The most probable explanation for these findings is that the calcium pump in this latter conformation has strong hydrophobic interactions and its calcium and ATP-binding hydrophylic domains, accessible to proteolysis in E_1 state, 'sink' into the hydrophobic membrane environment.

Interestingly, vanadate has been reported to have some protective effect on the proteolytic degradation of the isolated calcium pump as well [8]. In this preparation probably the detergent molecules attached to the pump simulate the hydrophobic action of the membrane lipids. A similar action of vanadate has been recently reported for the calcium pump in the sarcoplasmic reticulum membranes, where the second cleavage of the 55 kDa tryptic fragment of the pump was inhibited by EGTA + vanadate [17]. Most recently Andersen et al. [18] demonstrated major changes in the conformation of the sarcoplasmic reticulum calcium pump, as revealed by limited proteolysis of the E_1 and E_2 forms of the enzyme, respectively.

The results presented in this paper indicate that the E_1-E_2 conformation change of the red cell membrane calcium pump represents a major molecular alteration reflected in the proteolytic accessibility of the transport protein. These molecular conformation changes are best seen if the digestion of the basic pump unit, the 80 kDa fragment is followed. This peptide has already lost the easily cleavable hydrophilic groups which have no apparent role in the process of calcium translocation but rather in the regulation of the transport system.

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