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Identification of a cross-linked double-peptide from the catalytic site of the Ca^{2+}-ATPase of sarcoplasmic reticulum formed by the Ca^{2+}- and pH-dependent reaction with ATP γP-imidazolidate

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The Ca^{2+}-ATPase from sarcoplasmic reticulum can be inhibited by the Ca^{2+}- and pH-dependent reaction with ATP γP-imidazolidate. The chemically and monofunctionally activated inhibitor introduces an intramolecular cross-link between two neighbouring peptides of the active site. This can be followed by the reduced mobility of the ATPase upon SDS-PAGE analysis which becomes even more pronounced after limited trypsinolysis. After cleavage of the cross-linked ATPase molecule by cyanogen bromide and separation of the peptides a double-peptide can be detected which upon sequencing can be identified as part of the phosphorylation and the nucleotide binding site, respectively.

Ca^{2+}-ATPase; Sarcoplasmic reticulum; ATP γP-imidazolidate; Intramolecular cross-linking; Double-peptide, Catalytic site

1. INTRODUCTION

The Ca^{2+}-ATPase (EC 3.6.1.38) of sarcoplasmic reticulum uses the free energy produced by hydrolysis of MgATP for protein conformational changes which enable vectorial movement of Ca^{2+} across the membrane. The idea of how this might occur at the molecular level has taken shape with the knowledge of the primary structure [1] and the two-dimensional models of the Ca^{2+}-pump published recently [1–3].

Several amino acid side chains involved in the formation of the environment of the nucleotide binding site(s) have been identified, e.g. Asp^{351} [4] which becomes phosphorylated by the transfer of the γ-phosphate residue from ATP, Lys^{515} as the site of reaction with FITC which inactivates ATPase activity [5], Lys^{492} which has been found to react with adenosine 5′-triphosphopyridoxal (AP_3PL) in the absence of Ca^{2+} [6] and with TNP-8-N_3-ATP [7], respectively, and Lys^{684} which is the site of reaction with AP_3PL both, in the presence [8] and absence of Ca^{2+} [6]. Concerted action of these and possibly more amino acid side chains results in catalysis of ATP hydrolysis and concomitant phosphorylation/dephosphorylation which initiate the necessary conformational changes for Ca^{2+} transport.

We have previously shown [9] that an intramolecular cross-link can be introduced into the Ca^{2+}-ATPase of sarcoplasmic reticulum by the interaction with monofunctionally derivatized ATP and ADP. In a two-step reaction mechanism the nucleotides, activated at the end-phosphate group, form intermediate mixed anhydride bonds with Asp^{351} which serve as targets for the nucleophilic attack of a neighbouring amino acid side chain to replace the nucleotide. This second step of the reaction mechanism is dependent on the presence of calcium ions which probably reflects a conformational change in the environment of this amino acid residue normally taking place during the catalytic cycle. It covalently connects two amino acid side chains which represent a small, but very important part of the tertiary structure of the Ca^{2+}-ATPase. We have separated the peptides produced by cyanogen bromide digestion of intramolecularly cross-linked Ca^{2+}-ATPase by Tricine-SDS-PAGE [10] and sequenced the double-peptide newly formed and detected.

2. MATERIALS AND METHODS

2.1 *Materials*

ATP and ADP were from Boehringer Mannheim GmbH (Mannheim), Dowex ion-exchange resin type 50 WX8 p.a. (counterion H^+) was obtained from Serva (Heidelberg). [α-^{32}P]ATP and $^{45}CaCl_2$ were purchased from NEN Research Products, DuPont (Dreieich). Acrylamide and bis-acrylamide were from Carl Roth GmbH (Karlsruhe). Tricine, trypsin and trypsin inhibitor were obtained from Sigma GmbH (Deisenhofen). Immobilon (PVDF)-membranes were

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Abbreviations SR, sarcoplasmic reticulum; AP_3PL, adenosine 5′-triphosphopyridoxal; TNP-8-N_3-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azido-ATP.

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from Millipore (Eschborn). All other chemicals and solvents were of purest grade available from E. Merck (Darmstadt).

2.2. *Methods*

Sarcoplasmic reticulum vesicles were prepared from hind leg muscles of rabbit according to Hasselbach and Makinose [11] as modified by De Meis and Hasselbach [12]. Each preparation was characterized by measuring ATPase and Ca^{2+}-transporting activity and by SDS-PAGE according to Laemmli [13].

Intramolecular cross-linking of the ATPase in the presence of Ca^{2+} was induced by adding ATP γP-imidazolidate, which was synthesized according to Bill et al. [9], at the pH and pCa^{2+} indicated in the figure legends.

Limited trypsinolysis was performed at 0°C according to Stewart et al. [14], cyanogen bromide digestion as described by Zingde et al [15] and peptide separation achieved with a PAGE system developed by Schägger and von Jagow [10].

The peptide fragments were transferred onto Immobilon (PVDF)-membranes by the Western-blotting technique [16], briefly stained by Amidoblack, the new peptide band (compared to a digestion of non-cross-linked ATPase) cut out and directly sequenced with a 470 A Sequencer (Applied Biosystems) with online-HPLC of the resulting PTH-amino acids. The sequences found were checked with the aid of MIPS-data bank (Martinsried Institute for Protein Sequences)

3. RESULTS AND DISCUSSION

Since the intramolecular cross-link introduced into the tertiary structure of the ATPase molecule can be detected and its formation conveniently monitored by SDS-PAGE we investigated the yield of cross-linking in dependence on the pH and the pCa by this method. The aim was to find out whether the highest yield coincides with the optimum of Ca^{2+}-ATPase inhibition [9] in both cases or if there is any deviation from the behaviour obtained by these inactivation studies [9]. Furthermore a higher cross-linking yield should provide more material for sequencing purposes. Fig. 1 shows that the stained band of intramolecularly cross-linked Ca^{2+}-ATPase first appears weakly at 1 μM Ca^{2+}, becomes most prominent at a Ca^{2+} concentration of 10 μM, and decreases again in intensity at 100 μM Ca^{2+}. It does not form at all any more at 1 mM Ca^{2+}. This is in accordance with the results of inactivation studies where, at a concentration of 1 mM ATP imidazolidate, an optimal inhibitory effect is obtained at 10 μM Ca^{2+} [9]. It indicates that the sensitivity towards Ca^{2+} of the local conformational change which leads to cross-link formation is restricted to a relatively narrow concentration range. It also shows that the Ca^{2+}-induced conformational change is responsible for the readiness of the second amino acid side chain to replace the nucleotide by nucleophilic attack at the mixed anhydride bond which leads to inactivation of the Ca^{2+}-pump.

Such a nucleophilic reaction has to be dependent on the pH if an amino acid side chain with a pK within the physiological pH range is involved. Fig. 2 shows the formation of the cross-linked ATPase band on SDS-PAGE in dependence on the pH. The intensity of the new ATPase band varies with the pH as expected and is strongest between pH 7 and 8. It is also already detectable at pH 6.5 and still at pH 8.5 and 9 although much weaker. The ratio of the band intensities 'cross-linked ATPase/ATPase' gives a bell-shaped curve with a maximum at pH 7.5 (not shown) which coincides with the optimum of ATPase activity measured under our conditions [9]. It is an additional indication of the participation in the catalytic cycle of the amino acid side

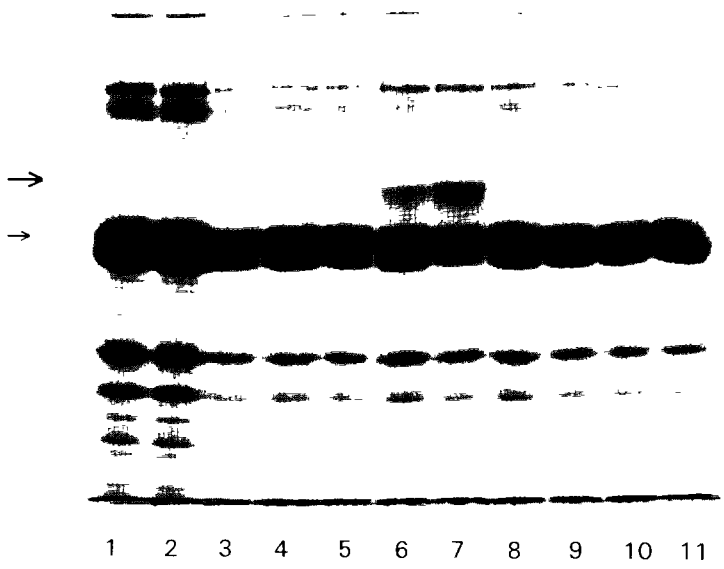

Fig. 1. Ca^{2+} dependence of cross-linked Ca^{2+}-ATPase formation. SR vesicles were reacted with 1 mM ATP γP-imidazolidate in the presence of different Ca^{2+} concentrations as indicated under the lanes and subjected to SDS-PAGE. The small arrow indicates the position of the Ca^{2+}-ATPase (110 kDa), the longer arrow that of the cross-linked protein (130 kDa). Control SR vesicle proteins (1) and (2). SR vesicle protein reacted in the absence (3) and the presence of 10 mM (4), 1 mM (5), 0.1 mM (6), 10 μM (7), 1 μM (8), 0.5 μM (9), 0.1 μM (10), and 0.01 μM (11) Ca^{2+}.

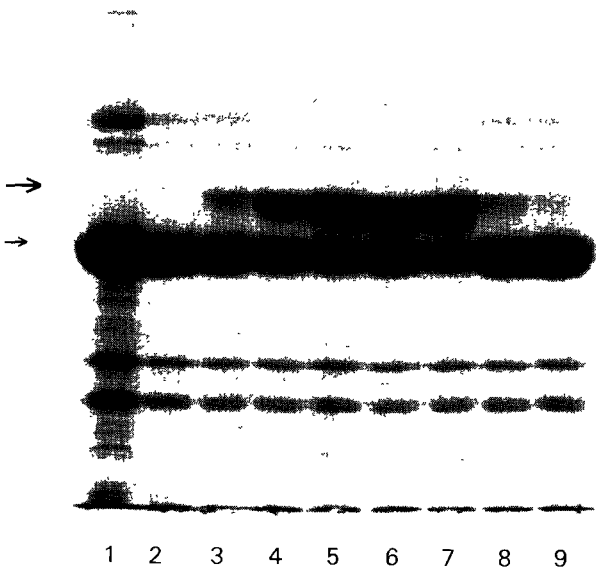

Fig. 2. pH dependence of cross-linked Ca^{2+}-ATPase formation. SR vesicles were treated with 1 mM ATP imidazolidate in the presence of 50 μM Ca^{2+} and the pH indicated and separated by SDS-PAGE. Control SR protein (1), pH 6 (2), pH 6 5 (3), pH 7 (4), pH 7 25 (5), pH 7.4 (6), pH 8 (7), pH 8.5 (8), and pH 9 (9). Arrows point to the position of the 110 kDa Ca^{2+}-ATPase and the 130 kDa cross-linked Ca^{2+}-pump

chain involved in the second step of the cross-link formation.

Since the intramolecular cross-linking of the Ca^{2+}-ATPase molecule produced such a large change in the electrophoretic mobility upon SDS-PAGE it seemed likely that two peptides far apart in the primary structure of the ATPase become connected. The reason is that the covalent reaction of two amino acid side chains close together in the amino acid sequence would not induce such a significant alteration of the hydrodynamic properties of the ATPase/SDS-micelle. Stewart et al. [14] could show that upon limited trypsinolysis the ATPase molecule is split into fragments A and B with molecular weights of 55 and 45 kDa, respectively. We used their method to find out whether fragments A and B become cross-linked or the covalent reaction takes place within one of the fragments. Fig. 3 shows that noncross-linked ATPase is cleaved into the fragments whereas cross-linked ATPase does not produce cleavage fragments A and B. On the contrary limited trypsinolysis leads to a further shift to higher molecular weight (approximately 140 kDa) probably representing an additional change of the ATPase/SDS-micelle. In Fig. 3 we have included a model proposing our idea of how the ATPase molecule might change after limited trypsinolysis. Similar results of intramolecular cross-linking at the active site of the Ca^{2+}-ATPase have been obtained with glutaraldehyde [17] and a carbodiimide adduct of ATP [18].

For sequencing purposes we used the above optimized conditions to prepare the cross-linked ATPase starting from 4 mg of sarcoplasmic reticulum protein. The detection and isolation of a double-peptide as a

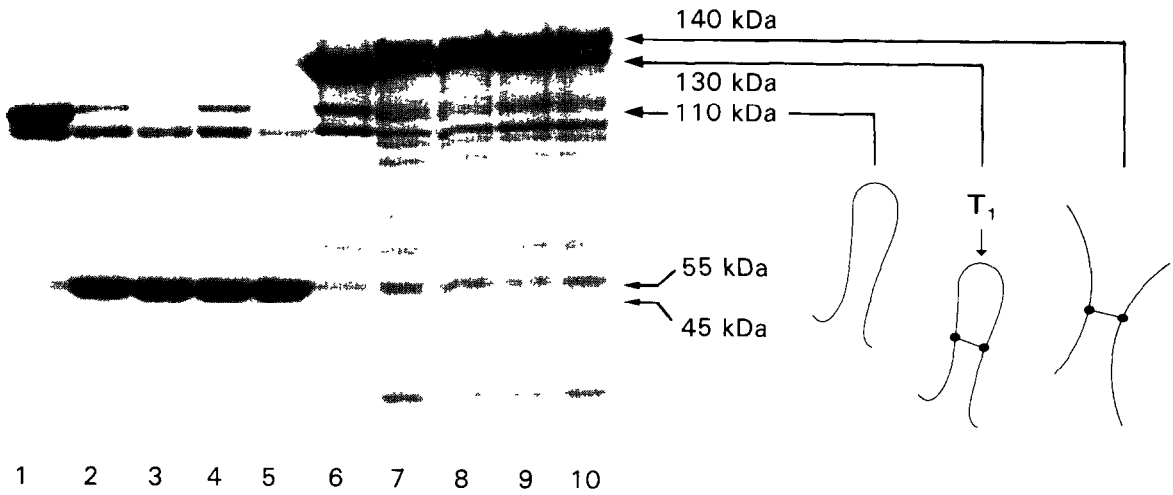

Fig. 3. Limited trypsinolysis of ATP imidazolidate treated and control SR vesicles. Non-cross-linked (2–5) and cross-linked (7–10) SR vesicles were subjected to limited trypsin digestion at 0°C for different lengths of time and analyzed by SDS-PAGE. The 110 kDa band indicates the position of untreated control Ca^{2+}-ATPase, the pair of small arrows the hardly separated trypsin T_1-cleavage peptides A and B (55 and 45 kDa) and the large arrows the positions of control cross-linked and trypsinolyzed cross-linked ATPase, respectively (130 and 140 kDa). Non-cross-linked control vesicles (1), 1 min (2), 5 min (3), 2.5 min (4), and 10 min (5) digestions of control SR Cross-linked SR vesicles (6), and 1 min (7), 2.5 min (8), 5 min (9), and 10 min (10) digestions of cross-linked SR vesicles.

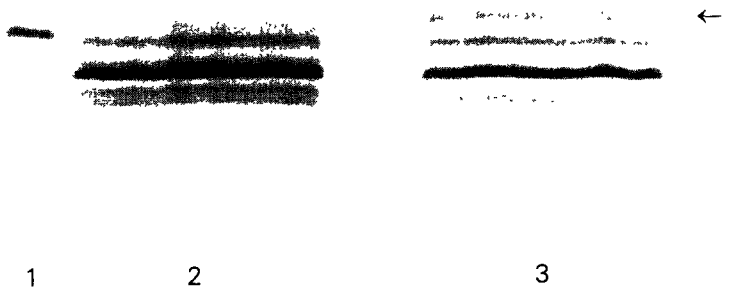

Fig. 4. Comparison of cyanogen-bromide cleavage products obtained from cross-linked and non-cross-linked Ca^{2+}-ATPase. ATP imidazolidate treated and untreated Ca^{2+}-ATPase reeluted from SDS polyacrylamide gels (7.5%) and precipitated according to [19] were subjected to cyanogen bromide cleavage and the peptides analyzed by tricine-SDS-PAGE. Marker proteins (1), non-cross-linked ATPase (2), and cross-linked ATPase (3). The arrow indicates the position of the newly formed double-peptide (ca. 12.4 kDa)

product of enzymatic digestion of the Ca^{2+}-ATPase was obstructed by the fact that during the reaction sequence of the ATPase with [^{32}P]ATP imidazolidate the radioactively labelled nucleotide leaves the protein due to the nucleophilic substitution reaction with a second amino acid side chain. Our strategy therefore was to look for a newly appearing double-peptide after cyanogen bromide digestion and separation of peptides on a gel system which had been especially developed for peptides down to a molecular weight of 1,000 Da [10]. There was no danger of losing smaller peptides possibly participating in the formation of the double-peptide since the cyanogen bromide peptide containing the phosphorylation site Asp^{351} is involved which on its own has a molecular weight of about 3,800 Da. Cross-linked and non-cross-linked Ca^{2+}-ATPase were separated by SDS-PAGE according to Laemmli [13]. The stained protein was re-eluted from cut out gel pieces by applying an electro-elutor from Schleicher and Schüll (Biotrap BT 1,000) and the protein subsequently precipitated according to [19]. After cyanogen bromide cleavage peptide samples were applied to Tricine-SDS-PAGE [10]. Fig. 4 shows the comparison of samples of both, cross-linked and non-cross-linked Ca^{2+}-ATPase, respectively. A prominent new peptide band of molecular weight 12.4 kDa can be detected in the cross-linked sample which was transferred onto Immobilon (PVDF)-membranes by electroblotting and then sequenced. Each Edman degradation step showed two amino acids corresponding to two N-termini of the cross-linked double-peptide. As expected one peptide contains the phosphorylation site and reaches from Ala^{327} to Met^{361}. The other peptide is part of the nucleotide binding site [1] and reaches from Ile^{624} to Met^{700}. Fig. 5 shows the part of the double- peptide which contains the bridge between the two peptides. Their po-sition is in close proximity in the

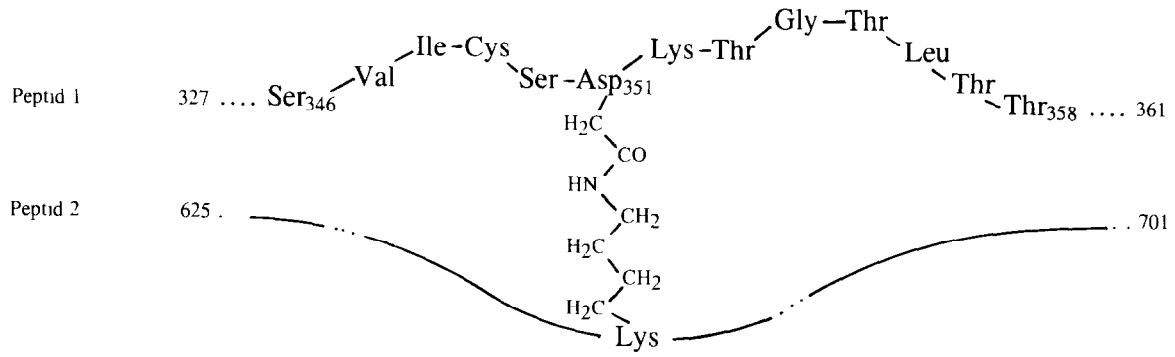

Fig. 5. Model of the double-peptide formed by intramolecular cross-linking. The model shows the participation in the formation of a cross-link of the peptide Ala^{327}–Met^{361} containing the phosphorylation site Asp^{351}. A lysine residue from peptide Ile^{624}–Met^{700} was chosen as the hypothetical reaction partner which forms a covalent bond with Asp^{351}.

tertiary structure according to the two-dimensional models of both, MacLennan and Toyofuku [3] as well as East et al. [2].

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