Intramolecular Cross-linking of Domains at the Active Site Links A1 and B Subfragments of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum

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Glutaraldehyde treatment of sarcoplasmic reticulum vesicles results in formation of cross-linked Ca^{2+}-ATPase oligomers. Under limiting reaction conditions, where minimal interpolypeptide cross-linking occurs, hydrodynamic properties of the monomer are altered, such that, on sodium dodecyl sulfate-polyacrylamide electrophoresis, the enzyme migrates with an apparent molecular weight of 125,000 (E(125)), as compared to the native enzyme (E(110)). The E(125) species was also formed following reaction with other cross-linking bis-aldehydes, with formaldehyde and with a bis-succinimidyl ester. Derivitization resulted in inactivation of ATPase activity and of phosphoprotein formation from Pi. E(125) formation was inhibited by ATP, ADP, AMPPCP, and orthovanadate, and by specific modification of active site Lys-514 with fluorescein-5'-isothiocyanate. Tryptic cleavage patterns of the glutaraldehyde-modified enzyme were consistent with covalent linkage of A1 and B fragments that have been postulated to comprise the phosphorylation and nucleotide-binding domains (MacLennan, D. H., Brandt, C. J., Korczak, E., and Green, N. M. (1985) Nature 316, 696-700). The denaturing detergent, sodium dodecyl sulfate, prevented cross-link formation. Interdomain cross-linking was inhibited by prior modification with either 2,4,6-trinitrobenzene sulfonate, phenylglyoxal, or pyridoxal-5'-phosphate but was unaffected by thiourea group modification with iodoacetate or N-ethylmaleimide, suggesting involvement of lysine residues. These findings indicate that intramolecular cross-linking at the active site of the Ca^{2+}-ATPase involves phosphorylation- and ATP-binding domains that are widely separated in the linear sequence.

The primary structure of the Ca^{2+}-ATPase of rabbit skeletal muscle sarcoplasmic reticulum (SR) has recently been determined and speculative models of the secondary and tertiary structures have been proposed (1). The protein is predicted to be composed of an intramembranous region, a stalk section, and three globular cytoplasmic domains. The enzyme has two particularly sensitive tryptic cleavage sites, T1 and T2, which fragment the polypeptide, initially to A (M_r = 55,000) and B (M_r = 54,000) peptides, and subsequently into A1 (M_r = 33,000) and A2 (M_r = 22,000). The cleavage sites approximately delimit the hypothetical cytoplasmic domains, so that A1 contains the phosphorylation site, and B the ATP-binding domain. It has been suggested that A2 constitutes an energy transduction domain (1). The nucleotide domain has been identified, in part, from localization of a lysyl residue (Lys-514), which is specifically modified by fluorescein-5'-isothiocyanate (FITC), resulting in inhibition of ATP-dependent catalytic function (2, 3). Another "essential" lysyl residue has been located on the A fragment, which contains the aspartate residue that is phosphorylated (Asp-351), through its specific reaction with pyridoxal-5'-phosphate and inhibition of ATPase activity (4).

It has been proposed that catalysis in dehydrogenases and kinases involves conformational changes that result in movements of separate domains with respect to each other about a "hinge region." This movement, triggered by substrate binding, brings the substrates together, enclosing them in an isolated active site where the reaction takes place. Reversal of domain movement releases the products, and the enzyme is ready for another catalytic event (for review see Ref. 5). In the case of the Ca^{2+}-ATPase, it can be predicted that the ATP-binding site on the nucleotide-binding domain, and Asp-351 on the phosphorylation domain, must be in close proximity: Segments of these two domains presumably contribute to formation of the active site.

Intramolecular cross-linking has been used as a method for investigating protein folding and tertiary structure. Introduction of such cross-links into ribonuclease A (6) and lysozyme (7) results in altered physical properties and increased thermal stability. Active site residues have been cross-linked in adenosine cyclic 3',5'-monophosphate- and guanosine cyclic 3',5'-monophosphate-dependent protein kinases (8, 9). In this paper we report on the effects of bifunctional agents on the Ca^{2+}-ATPase that result in intramolecular cross-linking, under conditions where minimal cross-linked oligomers are formed. The modified protein can be conveniently detected by its altered mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cross-linking appears to occur at the active site and to involve residues situated on domains widely separated in the linear sequence.

EXPERIMENTAL PROCEDURES

Materials—Glutaraldehyde (Grade II, 25% aqueous solution), hydrazine, N,N'-p-phenylenediaminodisulfonic acid, iodine, ethyl acetimidate, FITC, bovine pancreas trypsin, soybean trypsin inhibitor, pyridoxal-5'-phosphate, and Triton X-100 were purchased from Sigma. Triton X-100 was purified by the method of Askari and Catravas (10). Dimethyl suberimidate and ethylene glycol bis(succinimidyl succinate) were products of Pierce. Glyoxal was obtained from Fluka. p-
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Phenylendiglyoxal was from Molecular Probes, 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and phenylglyoxal were products of Aldrich. Iodoacetic acid, ethylamine, and formaldehyde were obtained from BDH Chemicals Ltd. Dodecyl octaethylene glycol monooether (C$$_{12}$$EO) was purchased from Nikkoil, through Kyouh Toyoh Trading Co., Tokyo, Japan. Urea, marker proteins, and gel electrophoresis chemicals were obtained from Bio-Rad.

SR vesicles were prepared from rabbit back and hind limb muscle by the method of Eletr and Inesi (11). These were stored as a suspension (10-20 mg of protein/ml) in 10 mM imidazole, pH 7.4, and 0.3 M sucrose at 0 °C or -60 °C and used within 4 days or two months. Protein concentrations were estimated by the Lowry method, using bovine serum albumin as standard and sodium deoxycholate as a solubilizing agent.

**Cross-linking**—Reaction of intact or detergent-solubilized SR vesicles (0.4 mg protein/ml) with various cross-linkers was carried out at 25 °C in the medium specified below the legends to the figures. The reaction times with glutaraldehyde and the glutaraldehyde concentrations of glutaraldehyde were calculated assuming a molecular weight of 125,000, with and without the glutaraldehyde concentrations were estimated by the Lowry method, using bovine serum albumin as standard and sodium deoxycholate as a solubilizing agent.

**Chemical Modifications**—SR vesicles (2 mg SR protein/ml) were chemically modified with various monofunctional reagents in the medium indicated below for the specified times at 25 °C. All reagent solutions were made up fresh prior to a short incubation in the stock was added to the SR protein to the final concentration indicated. (The methods for chemical modification of proteins with these various reagents were adapted from information on chemical reactions in Ref. 12, except for FITC and pyridoxal-5'-phosphate labeling.)

(i) TNBS, 30 mM MOPS/TEA, pH 8.5, 50 mM CaCl$$_2$$, and 2 mM TNBS for 60 min. (ii) FITC, 30 mM MOPS/TEA, pH 7.5, 1 mM EGTA, 2 mM MgCl$$\text{2}$$, and 25 mM FITC for 60 min (13). (iii) Ethyl acetimidate, 30 mM MOPS/TEA, pH 8.5, 50 mM CaCl$$\text{2}$$, 2 mM MgCl$$\text{2}$$, and 2 mM ethyl acetimidate for 60 min. (iv) Pyridoxal-5'-phosphate, 30 mM MOPS/TEA, pH 7.5, 50 mM MgCl$$\text{2}$$, 5 mM pyridoxal-5'-phosphate, 50 mM MgCl$$\text{2}$$, 5 mM pyridoxal-5'-phosphate, 50 mM MgCl$$\text{2}$$, and 5 mM pyridoxal-5'-phosphate for 10 min (4). (v) Controls, 30 mM MOPS/TEA, pH 8.0, 2 mM MgCl$$\text{2}$$, and either 50 mM CaCl$$\text{2}$$ or 1 mM EGTA for 60 min.

**RESULTS**

The protein content of SR vesicle preparations routinely contain about 80% Ca$$^{2+}$$-ATPase, with variable amounts of phosphophorylase (M$$\text{r}$, 93,000), calsequestrin (M$$\text{r}$, 60,000), and other unidentified but lower molecular weight proteins (Fig. 1A). The Ca$$^{2+}$$-ATPase has a calculated molecular mass of 109,771 daltons, based on the deduced primary sequence (1) and it bands close to this value, according to standard protein markers, in gradient gels. Reaction of SR vesicles with glutaraldehyde resulted in the formation of a protein species with an apparent molecular weight of 125,000, with a maximum molar excess of 0.3% Triton X-100 for 30 min at 37 °C. Thereafter, the samples were cooled to 25 °C and cross-linked. The enzyme exhibited no Ca$$^{2+}$$-dependent ATPase activity after the EGTA treatment.

The pH dependence of the formation of E(125) is shown in Fig. 1B. This behavior is consistent with reaction of an ionizable group with a pK$$\text{a}$ of approximately 7.5, and is maximum above pH 8.0. The observed pH dependence is not due to the lower reactivity of glutaraldehyde at acid pH values, since higher concentrations of glutaraldehyde, or longer reaction times at pH 6.0, resulted in increased amounts of cross-linked oligomers of ATPase but not in the amount of E(125) (not shown).

The effects of solubilization of SR vesicles in Triton X-100 (0.2%) and of a longer glutaraldehyde reaction time, together with the ability of various other cross-linking agents to form E(125), are shown in Fig. 2. The concentrations of monovalent and divalent cations are low in order to minimize protein-protein interactions. Reaction with glutaraldehyde under these conditions resulted in close to 100% loss of the native Ca$$^{2+}$$-ATPase and appearance of almost stoichiometric amounts of E(125). Other aldehyde-based cross-linkers, such as formaldehyde, glyoxal, and p-phenylenediglyoxal, as well as the bis-succinimidyl ester reagent, ethylene glycol bis(succinimidyl succinate), also formed E(125) but were not as effective as glutaraldehyde. The bis-inoimidoester, dimethyl suberimidate, did not cause conversion of E(110) to E(125), although in the absence of detergent this reaction produced cross-linked ATPase dimers, trimers, and other larger oligomers, indicating that the concentration of the reagent was not limiting. Oxidation of sulfhydryls with I$_2$, or reaction with the bifunctional sulfhydryl reagent, N,N' -p-phenylenedimaleimide, resulted in reaction with the ability of various other cross-linking agents to form E(125). E(125) is the apparent molecular weight, as determined by SDS-PAGE. The pattern of cross-linking and increasing ratio of E(125) to E(110), as the extent of reaction was increased, indicates that the majority of the ATPase cross-links first to E(125), before cross-linking to higher order oligomers.

**ATPase Activity**—ATPase activity was measured by the pyruvate kinase-lactate dehydrogenase coupled assay system with 5 mM ATP (15). The enzyme activity represents the Ca$$^{2+}$$-dependent component, obtained from the difference of the activity measured in the presence of calcium ions and that in calcium-free EGTA. The calcium ionophore, A23187 (0.05 μmol/mg of protein), was included to make the vesicles "leaky.

**E-P Measurements**—SR vesicle phosphorylation from P, was determined with $[^{32}P]P$, and a filtration procedure (16).
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FIG. 1. Reaction of glutaraldehyde with SR vesicles. Effect of glutaraldehyde concentration and pH on the banding pattern on SDS-PAGE. SR vesicles (0.4 mg of protein/ml) were reacted in (A) with glutaraldehyde, at the concentrations shown, for 4 min in a medium of 50 mM MOPS/TRIS, pH 8.0, and 50 μM CaCl\textsubscript{2} and in (B) with 5 mM glutaraldehyde for 4 min in a medium of 50 mM MES/MOPS/TRIS, pH 6.0, and 6.5, MOPS/TRIS, pH 7.0, 7.5, and 8.0, MOPS/CHES, pH 8.5, and 50 μM CaCl\textsubscript{2}. Untreated SR vesicles are shown in the first lane in both cases.

FIG. 2. Effect of various cross-linking agents on solubilized SR proteins as judged by their banding pattern on SDS-PAGE. SR vesicles (0.4 mg of protein/ml) were preincubated in 100 mM MOPS/TEA, pH 8.0, 50 μM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 30 mM KCl, and 0.2% Triton X-100 for 10 min at 25 °C. The glutaraldehyde (5 mM) reaction was carried out for 6 min. The concentrations of the other cross-linkers and the reaction times are specified under "Experimental Procedures." The lanes represent (A) control untreated SR vesicles, (B) glutaraldehyde, (C) glyoxal, (D) formaldehyde, (E) dimethyl suberimidate, (F) ethylene glycol bis(succinimidy1 succinate), (G) I\textsubscript{2}, (H) p-phenyldiglyoxal and (I) N,N'-p-phenylenedimaleimide.

FIG. 3. Effect of prior chemical modification of SR vesicles on the formation of E(125) with glutaraldehyde. SR vesicles (2 mg of protein/ml) were incubated with 50 μM CaCl\textsubscript{2} (lanes A and I) and 1 mM EGTA (lane B) in the absence of any chemical agents (controls) and 2,4,6-trinitrobenzoic acid (lane C), fluorescein-5'-isothiocyanate (lane D), ethyl acrylatide (lane E), phenyl glyoxal (lane F), iodoacetate (lane G), and pyridoxal-5'-phosphate (lane H) as described under "Experimental Procedures." The reaction mix was diluted 5-fold with 100 mM MOPS/TRIS, pH 8.0, and the concentration of CaCl\textsubscript{2} adjusted to 50 μM. The glutaraldehyde (5 mM) reaction was carried out for 4 min. Untreated SR preparations are shown in lanes labeled SR; that on the left of the gel is the preparation used for experiments A-G and that on the right for experiments H and I.

but distinct from that modified by FITC (4), as well as reaction with TNBS (lane C) or phenylglyoxal (lane F), inhibited or prevented the formation of E(125). Prior treatment of the Ca\textsuperscript{2+}-ATPase with ethyl acrylatide (lane E), under conditions described under "Experimental Procedures," had no effect on the formation of E(125). No E(125) was formed, however, following extensive modification with 100 mM ethyl acrylatide for 60 min but resulted in a large proportion of the protein cross-linking to high molecular weight aggregates that did not enter the running and stacking gels (not shown). Reaction with iodoacetate (Fig. 3, lane G) or N-ethylmaleimide (not shown) had no effect on the cross-linking reaction.
The effects of various nucleotides, p-nitrophenyl phosphate (pNPP) and orthovanadate on the formation of E(125) are shown in Fig. 4. In preliminary experiments, using thin layer chromatography to monitor the identity of the nucleotides, we determined that glutaraldehyde did not react with adenine nucleotides under these conditions. ATP, AMPPCP, ADP, ITP (each 0.5 mM) and orthovanadate (0.1 mM) substantially or completely prevented formation of E(125). AMP and pNPP (each 0.5 mM) caused partial inhibition. Phosphate (10 mM with 10 mM MgCl₂) had very little effect (not shown).

Reaction of glutaraldehyde with the Ca²⁺-ATPase resulted in a time- and pH-dependent inactivation of ATPase activity and of phosphoenzyme formation from Pi (Fig. 5, A and C). Although there was a linear relationship between the extent of inactivation and amount of E(125) species formed, the former was greater than the latter. This effect was more marked at pH 6.5 than at 8 (Fig. 5B). The effect of ATP on ATPase inactivation by glutaraldehyde is shown in Table I. ATP had little protective effect on hydrolytic activity but completely inhibited E(125) formation at both concentrations used.

Results of tryptic digestion experiments are shown in Fig. 6. Mild tryptic digestion of the Ca²⁺-ATPase resulted in the formation of fragments A (55,000) and B (54,000), followed by the cleavage of A into A₁ (33,000) and A₂ (22,000) (see lanes 6 and 8). Tryptic digestion of glutaraldehyde-treated SR vesicles produced a new protein band, migrating with an apparent molecular weight of 135,000, with loss of E(125) (lane 4). The residual unmodified protein (E(110)) was cleaved into A and B fragments. Further tryptic digestion resulted in conversion of E(135) into two fragments, one which comigrated with A₂ and the other with a molecular
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Effect of ATP on inactivation of ATPase activity by glutaraldehyde

SR vesicles (0.4 mg/ml) were preincubated at 25°C for 3 min in 10 mM MOPS/TRIS, pH 8.0, 0.5 mM EGTA, 5 mM glutaraldehyde, and at the concentrations of ATP indicated (added as an equimolar mixture with Mg$^{2+}$). The reaction was stopped with 100 mM hydrazine, and aliquots were taken for measurement of the Ca$^{2+}$-dependent component of ATPase activity using a coupled assay. Control 1 and 2 represent untreated SR vesicles without and with pre-reacted glutaraldehyde and hydrazine, respectively.

| Concentration of ATP (mM) | Activity (μmol/min/mg) |
|---------------------------|------------------------|
| 0 (control 1)             | 3.59                   |
| 0 (control 2)             | 3.14                   |
| 0.1                       | 1.42                   |
| 0.5                       | 1.66                   |

The reaction was stopped with 100 mM hydrazine, and aliquots were taken for measurement of the Ca$^{2+}$-dependent component of ATPase activity using a coupled assay. Control 1 and 2 represent untreated SR vesicles without and with pre-reacted glutaraldehyde and hydrazine, respectively.

Confirming this cleavage pathway is provided by the banding pattern which develops with time at constant trypsin concentration (Fig. 7). It is clear that the cross-linked enzyme, E(125), becomes A-B (E(135)) with the first cleavage, then A$_1$-B (E(108)) and A$_2$ on the second. Very little A$_4$ was formed. It also appears that A$_1$-B is converted to E(102) on further digestion and that this is most likely due to cleavage at a site on the A$_4$ fragment (18). An interesting observation is that solubilization of the tryptic fragmented Ca$^{2+}$-ATPase in Triton X-100 (0.5%) did not prevent the cross-linking of A and B or A$_1$ and B fragments, indicating a tight association between the fragments (not shown). It has been reported that the tryptic fragments cannot be dissociated in non-ionic detergents (19).

The effects of various treatments of the SR preparations on cross-linking with glutaraldehyde is shown in Fig. 8. Barrabini et al. (17) have reported that passage of SR protein through DEAE-cellulose in C$_{12}$E$_6$, and elution of the Ca$^{2+}$-ATPase with salt, results in a purification and selective enrichment of active over inactive ATPases. We found that most contaminating proteins, with a molecular weight lower than that of the ATPase, are lost with this procedure, but that some higher molecular weight species become evident (lane B). The purification procedure had little effect on the proportion of E(125) formed by the glutaraldehyde reaction (compare lanes C and D). It is estimated that approximately 80% of the ATPase was converted to E(125) in both purified and unpurified preparations.

Concentrations of C$_{12}$E$_6$, up to 1%, resulted in slightly broader bands but had little effect on the formation of E(125) (lane E). The same concentration of Triton X-100 produced less sharp and less intensely staining protein bands, but substantial E(125) was still evident (lane F). Prior solubilization of SR in SDS (lane G) or deoxycholate (not shown) prevented cross-linking with glutaraldehyde. Inactivation of the enzyme by incubation in Triton X-100 with excess EGTA also prevented the cross-link formation (lane H), although substantial dimer was evident. Aging of the preparation, which results in inactivation of Ca$^{2+}$ transport but not of ATPase activity (20), did not inhibit the cross-link (lane I).

**DISCUSSION**

We have shown, in this series of experiments, that several bis-aldehydes, formaldehyde, and the bis-succinimidyl ester, ethylene glycol bis(succinimidyl succinate) react with SR vesicles, intact or solubilized in non-ionic detergent, to produce a protein species, E(125), on SDS-PAGE, with an apparent molecular weight of 125,000. The concomitant disappearance of the Ca$^{2+}$-ATPase band (M = 110,000) and the relatively high proportion of Ca$^{2+}$-ATPase protein in the preparation (≈80%) indicates that E(125) is derived from the Ca$^{2+}$-ATPase. Several results suggest that the increase in apparent molecular weight of the Ca$^{2+}$-ATPase is not due to cross-linking to a small membrane protein. (a) The SR preparation does not contain low molecular mass proteins in the range 13–36 kDa in sufficient amounts as visualized on SDS-PAGE by Coomassie Blue staining (see Fig. 1) or by the more sensitive silver stain (not shown). However, the possibility cannot be excluded that small peptides, below about 10 kDa, that are not well resolved by SDS-PAGE could cross-link to the Ca$^{2+}$-ATPase. (b) The conversion is insensitive to purification of the Ca$^{2+}$-ATPase on DEAE-ion exchange chromatography. (c) The percentage conversion of Ca$^{2+}$-ATPase into the modified protein can be increased to nearly stoichiometric amounts with longer reaction times in C$_{12}$E$_6$ or Triton X-100, under conditions in which interpolyepitide contact is minimized. (d) Tryptic cleavage patterns of the modified enzyme...
Fig. 7. Time dependence of tryp- tic cleavage following reaction with glutaraldehyde. Modification with glutaraldehyde and the conditions for trypsinization (1:10 (w/w) trypsin to SR protein) are given in Fig. 5 and under "Experimental Procedures." The time of digestion is as indicated.

Fig. 8. Effect of purification, solubilization at high detergent concentrations, EGTA inactivation of ATPase activity, and aging on the formation of E(125). Untreated and a DEAE-treated SR preparation (see "Experimental Procedures") are shown without cross-linking in lanes A and B, respectively. In lanes C-I different preparations were cross-linked with two consecutive additions of 5 mM glutaraldehyde for 2 min each. Untreated SR vesicles (0.4 mg of protein/ml) (lanes C, E-G) were preincubated for 20 min at 25 °C in 100 mM MOPS/TRIS/CHES, pH 8.5, 100 μM CaCl₂, 50 mM NaCl, and either 0.3% C₁₂E₈ (lane C), 1% C₁₂E₈ (lane E), 1% Triton X-100 (lane F), or 0.2% SDS (lane G), and then cross-linked. The DEAE-treated SR preparation shown in lane B and a standard SR preparation aged for 7 days at room temperature with 0.02% NaN₂, were preincubated as above with 0.3% C₁₂E₈ and then cross-linked (lanes D and I, respectively). An EGTA-inactivated SR preparation (see "Experimental Procedures") was cross-linked (lane H).

Indicate that a cross-link is formed between regions delimited by tryptic fragments A₁ and B.

The evidence is consistent with formation of a cross-link between the A₁ and B fragments, due to glutaraldehyde reacting at residues in the active site. Nucleotide binding to the active site, at concentrations known to saturate this site (0.5 mM ATP, ADP, AMPPCP, and ITP), prevents cross-linking; AMP and pNPP (0.5 mM), which are known to bind with low affinity, caused some inhibition. Effects of orthovanadate binding, which results in a tight complex with the enzyme and mimics the transition state of the phosphorylation reaction with P₁ (21-24), suggests that phosphorylation or occupation of the active site by phosphate could prevent the cross-link. P₁ (10 mM) binding, however, had little effect and this may be due to the unfavorable pH for the phosphorylation reaction (25). Modification of Lys-514 on the B fragment by FITC, which is considered to be at the nucleotide-binding site (1-3), inhibited the cross-link.

Pyridoxal-5′-phosphate, which reacts specifically with a lysyl residue on the A₁ fragment, and which is also considered to be at the active site (4), also prevented cross-linking. The greater degree of functional inactivation of the enzyme (Fig. 5B) than expected from the extent of E(125) formation may be explained by a mechanism in which 2 residues at the active site need to be linked in E(125) but that modification of only 1 residue leads to an inactive enzyme. The marked pH dependence of derivitization and the disparity at more acid pH values suggests that the 2 residues involved may have different pKₐ values. It is interesting that ATP binding affords little protection against inactivation of the enzyme by glutaraldehyde and yet prevents the cross-link. Possibly, ATP binding only blocks reaction with one of the reactive residues and reaction with the other inactivates the enzyme.

The reaction of glutaraldehyde with proteins is complex and appears to involve the side groups of lysine, cysteine, histidine, and tyrosine (12). The best characterized reaction is Schiff base formation between carbonyls and primary amines (12). Carbonyl reactions with other functional groups...
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![Diagram](image)

**Fig. 9.** Effect of a hypothetical cross-link between Lys-352 and Lys-514 of the Ca\(^{2+}\)-ATPase on the extended forms of the uncleaved and T\(_1\)- and T\(_2\)-cleaved polypeptides. The arrangement of the polypeptide in the membrane, shown on the left, has been drawn according to predictions made by MacLennan et al. (1). The positions of Lys-352 and Lys-514 in the major cytoplasmic loop are shown as dots, and the first and second tryptic cleavage sites are marked by T\(_1\) and T\(_2\), respectively. The polypeptides, cross-link, and membrane have been drawn to scale. Apparent molecular weights found by SDS-PAGE and actual molecular weights (in parentheses) are shown below the putative structures.

have not been fully substantiated. Involvement of lysyl residues is suggested by the observation that bis-aldehydes formed the cross-link, as did the bis-succinimidyl ester, ethylene glycol bis(succinimidyl succinate). These reagents react almost exclusively with the \(\epsilon\)-amino group of lysine. Glyoxal, although considered to be fairly specific for arginyl residues, also reacts with lysyl residues (12). The lysine specific bis-imidoester cross-linking reagent, dimethyl suberimidate, did not, however, react with the Ca\(^{2+}\)-ATPase to form any E(125), although in the absence of Triton X-100, higher order cross-linked oligomers were evident. The imidoester functional group is positively charged and quite possibly could have limited access to a hydrophobic pocket, or could be repelled by positively charged regions of the protein, where the cross-link forms. Formaldehyde reacts with a wide range of functional groups in an undefined manner (12). It was difficult to conclude if any E(125) species was formed by sulfhydryl cross-linking with \(\epsilon\) oxidation or reaction with \(N,N\)\(p\)-phenylene dimaleimide.

The finding that prior chemical modification with (a) TNBS, which is specific for lysyl and cysteinyl residues, or (b) phenylglyoxal, which reacts with both arginyl and lysyl residues, and (b), the specific modifications of lysyl residues by FITC (Lys-514) and pyridoxal-5'-phosphate (Lys-\(\gamma\) on the A\(_1\), tryptic fragment), prevent the glutaraldehyde cross-link, also implicate lysyl residue involvement in the cross-link. Prior modification with iodoacetic acid and \(N\)-ethylmaleimide, both specific for sulphydryl groups, which did not prevent E(125) formation, indicate that cysteinyl residues are not involved. Several buried amino groups on the Ca\(^{2+}\)-ATPase have been shown to be unreactive to methyl acetoimidate (26) and may explain the ineffectiveness of the ethyl derivative in blocking E(125) formation. It is therefore suggested that lysyl residues, one close to the aspartyl residue on the A\(_1\) fragment that is phosphorylated by ATP or P\(_i\) (Asp-351), and one close to Lys-514, are involved in the cross-link. It is also possible that more than one type of cross-link could produce E(125).

Altered hydrodynamic properties of a protein on SDS-PAGE with resulting increase in apparent molecular size with the introduction of an intramolecular cross-link and further increase in apparent size with a single cleavage by trypsin has not, as far as we are able to ascertain, been described previously. Proteins that contain disulfide linkages generally migrate on SDS-PAGE, under nonreducing conditions, with lower apparent molecular size, compared with the reduced protein (27). This occurs despite decreased SDS binding in the unreduced, compared with the reduced state (28). Decreased frictional resistance is evidently the dominant factor affecting migration behavior. Reduced proteins assume an extended shape in SDS, the length of which varies in direct proportion to their molecular weight (29). Most intrapolypeptide disulfides form loops of about 50 residues or less, and the observed increase in electrophoretic mobility of unreduced proteins with intrapolypeptide disulfide linkages is simply explained by the decrease in extended length. Much longer loops are possible with artificial cross-links in large proteins, which would cause the shape of the polypeptide in SDS to deviate from the usual rod shape. This may compensate, in terms of frictional resistance, for the decrease in extended size and coupled with decreased SDS binding, could produce a species with an increase in apparent molecular weight on SDS-PAGE.

The relationship between the formation of the cross-link, tryptic cleavage, and apparent and actual molecular weights is shown diagrammatically in Fig. 9. A hypothetical cross-link is introduced at Lys-352 and Lys-514, for illustrative purposes and a relatively large loop of 162 residues would be formed by this connection. The reasons for choosing these particular residues are that they are on the correct trypsin subfragments and Asp-351 and Lys-514 are the only residues, thus far, which have been identified as being at the active site. The native polypeptide migrates in the expected position on SDS-PAGE according to its actual molecular weight of approximately 110,000 and evidently assumes the usual extended rod shape in SDS. The cross-link, and resulting loop, cause the polypeptide to exhibit an apparent molecular weight of...
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125,000. According to this model, T₁ cleavage produces a bifurcated molecule with an apparent molecular weight of 135,000. The “arms” produced by the cleavage evidently increase the frictional resistance of the polypeptide in SDS. T₂ cleavage produces an unaltered A₁ fragment and a polypeptide with an apparent molecular weight of approximately 108,000 and actual molecular weight of 88,000. This deviation is the same as that found following T₁ cleavage, and the structure shown in Fig. 9 has a distinct “Y” shape.

Formation of a loop by intramolecular cross-linking, of the size indicated in Fig. 9, is rather unlikely within a single protein domain. However, it is possible if cross-linking occurs between domains. MacLennan et al. (1) have recently proposed, from the predicted secondary structure of the enzyme, that the A₁ and B fragments contain the separate phosphorylation and nucleotide-binding domains, respectively. They are envisaged to consist of two parallel β-domains, connected by a hinge region, similar to that of phosphoglycerate kinase and hexokinase. In this model ATP-bound to the nucleotide-binding domain in fragment B, would phosphorylate the aspartic acid residue in fragment A₁ in the adjoining phosphorylation domain. Our proposal that the alteration in apparent molecular weight is due to the formation of a rather large loop is consistent with the phosphorylation- and ATP binding-sites being located in separate domains. In addition, the effectiveness of short cross-linkers, such as formaldehyde, suggests that the cross-link occurs close to the proposed hinge region or another region where the domains have close contact. Significantly, binding of Ca²⁺, which promotes the transition and reaction times are similar (results not shown). This also indicates that detergent does not uncover ATPase activity to tryptic digestion (32). We will show in a further paper that the inhibition of the cross-link by nucleotides, and by phosphorylation, provides a simple and sensitive assay for nucleotide and analogue binding and of occupancy of the active site during turnover and nonturnover conditions.

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