Mechanism of Inhibition of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase by Active Site Cross-linking

IMPAIRMENT OF NUCLEOTIDE BINDING SLOWS NUCLEOTIDE-DEPENDENT PHOSPHORYL TRANSFER, AND LOSS OF ACTIVE SITE FLEXIBILITY STABILIZES OCCLUDED FORMS AND BLOCKS E\(_{1}\)-P FORMATION*

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Investigation of the properties of Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum cross-linked at the active site with glutaraldehyde showed that ATP binding affinity and rate of ATP-dependent phosphorylation and Ca\(^{2+}\) occlusion were decreased 2–3 orders of magnitude compared with the native enzyme. Cross-linkage had little effect on or marginally increased the rate of acetyl phosphate- and p-nitrophenyl phosphate-supported Ca\(^{2+}\) occlusion. Ca\(^{2+}\) binding or Ca\(^{2+}\)-induced changes in tryptophan fluorescence were unaffected. High levels of phosphoenzyme (up to 4 nmol/mg of protein) were obtained, with 2 mol of Ca\(^{2+}\) occluded/mol of E-P. Dephosphorylation and deocclusion occurred together at a slow rate (k = 0.01 s\(^{-1}\)) and were stimulated in a monophasic manner up to 20-fold by ADP. Cross-linking inhibited E\(_{1}\)-P formation from P\(_{i}\) in 30% (v/v) dimethyl sulfoxide by more than 95%. Induction of turnover of the native ATPase, under conditions designed to yield high steady state levels of E\(_{1}\) ~ P(2Ca), resulted in a 3–4-fold increase in reactivity of active site residues to glutaraldehyde. The results show that cross-linkage sterically impairs nucleotide binding, changing ATP and ADP into relatively poor substrates, slowing nucleotide-dependent phosphoryl transfer and Ca\(^{2+}\) occlusion and deocclusion. The forward reaction with smaller substrates is unaffected. Another major effect of the cross-link is to inhibit E\(_{1}\)-P formation, causing accumulation of E\(_{2}\) ~ P(2Ca) during enzyme turnover and preventing phosphorylation by P\(_{i}\) in the reverse direction. We suggest that occlusion and deocclusion of cations at the transport site of the native enzyme are linked to a two-step cleft closure movement at the active site and that the cross-link stabilizes occluded forms of the pump because it blocks part of this tertiary structural change. The latter could normally be propagated through linking helices to the distal side of the pump to destabilize the cations and open the transport sites to the lumen.

Ca\(^{2+}\) transport by the Ca\(^{2+}\)-ATPase of skeletal muscle sarcoplasmic reticulum (SR)\(^3\) requires coupling of active site events to the binding and movement of Ca\(^{2+}\) ions through the protein. Analysis of the primary structure of the protein suggests that the tertiary structure consists of three extra-membranous domains, two of which combine to form an active site cleft, linked to a membrane-spanning section via a stalk of helical segments (1, 2). Recent site-directed mutation results suggest that the two high affinity Ca\(^{2+}\)-binding sites are located in the membrane-spanning section, some distance from the active site (3). It is not clear how events at the active site trigger those in the membrane region. Numerous kinetic and physicochemical studies have indicated that protein conformational changes occur at several, if not all, steps of the catalytic cycle, and it can be envisaged that communication through the protein might occur by shifting the position of linking helices in a way similar to that observed in allosteric transitions of soluble proteins (4).

In this study, we explore the role of active site residues and tertiary structural movements in catalysis by investigating the catalytic properties of the Ca\(^{2+}\)-ATPase cross-linked at the active site with glutaraldehyde. The cross-link connects tryptic fragments A1 and B and its formation is blocked by ATP binding at the active site or phosphorylation to the E\(_{2}\)-P catalytic intermediate (5, 6). The intermediate, E\(_{2}\) ~ P(2Ca), is the most reactive to glutaraldehyde, as we will show in this paper, and the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free conformations are approximately 3-fold less reactive. Thus, in terms of the reactivity of the active site residues, at least three different conformations of the active site exist. We also show here that cross-linking markedly lowers the affinity of the enzyme for ATP, slows ATP- and ADP-dependent phosphoryl transfer, stabilizes E\(_{2}\) ~ P(2Ca), and inhibits E\(_{2}\)-P formation in both directions of catalysis. The cross-link apparently sterically impairs nucleotide-dependent reactions as well as blocking a movement at the active site that is required for cation release to the lumen and E\(_{1}\)-P formation.

EXPERIMENTAL PROCEDURES

Materials—Glutaraldehyde (grade 1, 25% solution), ATP, acetyl phosphate, A23187, and p-nitrophenyl phosphate were purchased from Sigma.

SR vesicles were prepared from the back and hind leg muscles of fasted rabbits essentially according to the method of Eletr and Inesi (7), except that 0.1 mg of amylase (final concentration, 0.1 \(\mu\)g/ml) was added to the preparation during the initial homogenization step. This procedure eliminated the presence of phosphorylase and a protein of approximately 140 kilodaltons in the final SR preparation, and was found not to affect Ca\(^{2+}\) transport activity. The preparation was stored as before (5).

Cross-linking—The standard procedure for preparing cross-linked ATPase was to incubate SR vesicles (0.5 mg of protein/ml) for 70 min at 25 °C in 50 mM MOPS/triethanolamine, pH 8.1, 50 \(\mu\)M CaCl\(_{2}\),

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The abbreviations used are: SR, sarcoplasmic reticulum; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylenediaminetetraacetic acid; TES, Tris(hydroxymethyl)amino- methane; E(125), enzyme with \(M_{r} = 125,000\); E(110), enzyme with \(M_{r} = 110,000\); TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AMPPCP, adenosine 5’-(3,\(\gamma\)-methylene)triphosphate.
10 mM KCl, 0.2 mM sucrose, and 150 μM glutaraldehyde (27,000-fold dilution of 25% solution). Control preparations were incubated in the same way except that the glutaraldehyde was omitted. The suspension was then placed on ice and used within a few minutes. In some cases, the pH was adjusted to 7.0 with maleic acid prior to being placed on ice. In some experiments (see under "Results") and in the figure legends, when required, 7.4, 0.1 mM nucleotide or triethanolamine sulfate was included in the medium.

The reactivities of native ATPase to glutaraldehyde in the presence and in the absence of acetyl phosphate were carried out at room temperature in the medium described in the legend to the appropriate figure. Concentrations of glutaraldehyde were 1.5 mM, and 1.0 mM, respectively. Incubation was stopped after 2 min, and the reaction was stopped by adding 5 mM MgCl₂ and 2-fold dilution in 0.2 M MOPS/Tris, pH 7.4, 5 mM MgCl₂, pH 6.8, 3% (w/v) sodium dodecyl sulfate, 8 mM urea, and 4% (v/v) 2-mercaptoethanol.

**Binding Assays—** Equilibrium Ca²⁺ and ATP binding were carried out using a filtration procedure (8). Glass-fiber (Whatman GF/F) and MF type (Millipore) (0.45 μm) filters were used for Ca²⁺ and ATP binding, respectively. The wet volume on the filters was determined using [H]sucrose in the medium. SR vesicles (in the cross-linking medium) were diluted 5-fold into 50 mM MOPS/Tris, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 1 mM [H]sucrose. Either [Ca⁴⁺]CaCl₂ (50 μM) and EGTA (variable concentrations) or [γ-³²P]ATP (variable concentrations) were added to give the free concentrations shown in the figure. Final concentrations are given in the legend to the figure. The suspension (3 ml) was kept for 1 min at room temperature and then filtered. Free concentrations of Ca²⁺ were calculated, taking into account pH and Mg²⁺ concentrations according to the method of Fabiato and Fabiato (9).

**Phosphorylation—** Phosphoenzyme levels were measured at room temperature (=20 °C) by filtration with glass fiber filters (Whatman GF/F) after acid quenching (10). Measurements performed in the presence of ATP and MgCl₂ were carried out as follows. SR vesicles (0.2 ml, containing 0.1 mg of protein) were mixed with 1 ml of 50 mM MOPS/Tris, pH 7.4, 5 mM MgCl₂, 1 mM EGTA, and various [γ-³²P]ATP concentrations. Final concentrations are shown in the legend to the figure. In the case of P₁-supported phosphorylation, SR vesicles (0.5 mg of protein/ml, in the cross-linking medium) were diluted 5-fold into a buffer so that the final concentrations were 80 mM MOPS/Tris, pH 7.0, 2 mM KC, 40 mM sucrose, and 5 mM CaCl₂. Variable concentrations of [γ-³²P]ATP were added to initiate phosphorylation. Final concentrations are given in the legend to the figure. The reactions were quenched after 30 s with the same acid mixture used above; the suspension was also processed in the same way. Blanks were determined with 1 mM EGTA in place of the CaCl₂.

**Dephosphorylation—** SR vesicles were phosphorylated as above in the presence of MgCl₂ and 0.025 mM [γ-³²P]ATP, EGTA, 2 mM, plus variable concentrations of MgADP (equimolar mixture of MgCl₂ and ADP) were added after 2 min, and at timed intervals, aliquots were taken for acid quenching as above. Final concentrations are given in the legend to the figure.

**Ca²⁺ Uptake—** Ca²⁺ occlusion and transport were measured as follows. SR vesicles (0.2 ml of 0.5 mg of protein/ml, in cross-linking medium) were mixed with 0.8 ml of 50 mM MOPS/Tris, pH 7.4, 100 mM KCl, 5 mM MgCl₂, and 0.05 mM CaCl₂ and placed on top of a glass fiber (Whatman GF/F) filter in a standard Millipore filtration system in the absence of a vacuum so that the suspension did not penetrate the filter. Ca²⁺ uptake was initiated by the addition of 1 ml of 50 mM MOPS/Tris, pH 7.4, 5 mM MgCl₂, 1 mM EGTA, and substrate. A vacuum was applied at various times, and the filters were washed with 5 ml of a wash medium (50 mM MOPS/Tris, pH 7.4, 5 mM MgCl₂, 100 mM KCl, and 1 mM EGTA). In the case of Ca²⁺ occlusion (see the appropriate figure), 4% (w/v) A23187 was added to the SR suspension prior to mixing with substrate. Final concentrations are the same as those used in the cross-linking procedure. Reaction times were taken as the period from mixing to the arrival of the wash medium. Blanks were performed in the absence of substrate. Filters were counted in 5 ml of Insta-Gel. The assay was performed at room temperature.

**Ca²⁺ Release—** Ca²⁺ uptake was performed as above with 0.025 mM ATP for 1 min. The SR vesicles were layered onto glass fiber filters under reduced pressure and then perfused with wash medium (see above) with or without ADP (2 mM) for the times shown in the figure. The filters were then assayed for radioactivity.

**ATPase Activity—** Hydrolysis of ATP was determined spectrophotometrically at 340 nm and 25 °C by a coupled enzyme assay in 50 mM MOPS/Tris, pH 7.4, 5 mM MgCl₂, 100 mM KCl, either 0.05 mM CaCl₂ or 1 mM EGTA, 4% (w/w) A23187, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.1 mM NADH, 0.1 mg/ml of both bactic dehydrogenase and pyruvate kinase, and approximately 0.005 mg of SR protein/ml. The "basal" rate in the presence of EGTA was subtracted from that in the presence of Ca²⁺ to obtain the Ca²⁺-stimulated component.

**Fluorescence Measurements—** Ca²⁺-induced changes in tryptophan fluorescence (λex = 290 nm and λem = 330 nm) were measured on an Amino spectrofluorimeter (model SPF-500) in a continuously stirred cuvette at room temperature. SR vesicles were diluted 5-fold into 50 mM MOPS/Tris, pH 7.0, 100 mM KCl, and 5 mM MgCl₂. CaCl₂ (0.2 mM) and EGTA (variable concentrations) were added to give the required concentrations of free Ca²⁺. Final concentrations are given in the legend of the appropriate figure. Calculations of free Ca²⁺ concentrations took into consideration the pH and Mg²⁺ concentrations (9).

**Polyacrylamide Gel Electrophoresis—** Electrophoresis was carried out using the method of Laemmli (11), with 7% acrylamide as described before (8). The protein bands were stained with Coomasie Blue and scanned either in a wet or dried form with a Vitatron TLD densitometer.

**RESULTS**

We have previously shown that glutaraldehyde reacts with SR vesicles to form an intramolecular cross-link at the active site of the Ca²⁺-ATPase (5, 6). This reaction is kinetically faster than the nonspecific intermolecular cross-linking to oligomers. The intramolecular cross-link is readily detected through the abnormal mobility of the modified protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The time dependence of the reaction of glutaraldehyde with SR vesicles, under conditions found to be optimal for maximizing intramolecular cross-linking and minimizing intermolecular cross-linking is shown in Fig. 1A. Conversion of the native ATPase, M, ≈ 110 kDa and denoted E(110), to the cross-linked form, M, ≈ 125 kDa and denoted E(125), is substantial after 30 min to 1 h of reaction, with formation of higher molecular weight oligomers occurring at a slower rate.

The reaction of glutaraldehyde to form E(125) species is first order under the conditions used (k = 0.046 min⁻¹) and is directly correlated, within experimental error, with inactivation of Ca²⁺-stimulated ATPase activity (k = 0.055 min⁻¹) and of phosphorylation from P, in a Ca²⁺-depleted medium in 30% dimethyl sulfoxide (k = 0.055 min⁻¹) (Fig. 1B). The slightly faster inactivation of enzyme activity compared with E(125) formation, if significant, may be due to a small contribution to inactivation through intermolecular cross-linking of ATPases. In the presence of 0.1 mM AMPPCP (results not shown), E(125) formation was inhibited over 95% after 70 min of reaction, and the ATPase activity was approximately 90% of the control value, providing additional evidence that the effects of glutaraldehyde on enzyme activity are mediated by the intramolecular cross-link at the active site. A reaction time of 70 min was chosen to prepare cross-linked Ca²⁺-ATPase preparations for the experiments reported below. Gel scans indicated that these preparations contained approximately 85% intramolecularly cross-linked ATPase, 13% oligomers, and 2% E(110) species.

Ca²⁺ and ATP binding to native and cross-linked ATPase are shown in Fig. 2. Ca²⁺ binding to cross-linked ATPase was similar to that of control ATPase. The cross-linked prepara-
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Fig. 1. Time dependence of $E_{125}$ formation and its effect on ATPase activity and phosphorylation by P. A, SR vesicles (6.5 mg of protein/ml) were reacted with glutaraldehyde for the times shown, and aliquots were taken for electrophoresis. B, the percentage of $E_{125}$ (triangles), which was calculated as $E_{125} = 100/[E_{110} + E_{125}]$, was obtained by a scan of the gel in A. Ca\textsuperscript{2+}-stimulated ATPase activity (closed circles) and phosphorylation from P\textsubscript{i} (open circles) were measured on aliquots taken during the glutaraldehyde reaction. P\textsubscript{i} phosphorylation was carried out in 56 mM MOPS/Tris, pH 7.0, 30% (v/v) dimethyl sulfoxide, 2 mM KCl, 10 mM MgCl\textsubscript{2}, 1 mM EGTA, 2 mM P\textsubscript{i}, 40 mM sucrose, and 0.083 mg of SR protein/ml. The data have been fitted to a first order process with concentration dependence and the value for the change in fluorescence was also slightly less (Fig. 2).

In Fig. 2B, the kinetics of Ca\textsuperscript{2+}-dependent phosphorylation of control enzyme is compared to that of preparations cross-linked to the extent of 90, 65, 40, and 5%, either by shortening the cross-linking time or by including nucleotide or sulfate in the cross-linking medium. Phosphorylation of the native ATPase and the nucleotide-protected ATPase was complete within 1 s, the shortest sampling time achieved with the manual procedure used. In contrast, the partially cross-linked preparations exhibited biphasic kinetics with the fast phase attributable to the uncross-linked ATPases and the slower phase to the cross-linked ATPases. The high stoichiometry of phosphoenzyme formation obtained with the cross-linked preparation, coupled with the correlation between the proportion of cross-linked ATPase and the proportion of slowly phosphorylatable species, indicates that the cross-link significantly slows nucleotide-dependent phosphorylation.

The kinetics and ATP dependence of phosphorylation in the presence of Mg\textsuperscript{2+} of a cross-linked preparation are shown in Fig. 3B. The rates of phosphorylation ($k_{obs} \approx 0.7 \text{s}^{-1}$ at saturation) were approximately 100-fold slower than the native ATPase ($k_{obs} = 85-220 \text{s}^{-1}$ at saturation) (12-14). The $K_{m}$ (98 $\mu$M, Fig. 3B, inset) was higher than that of the native enzyme (10–20 $\mu$M) (13, 14), consistent with the lower affinity for ATP, demonstrated above. A $K_{m}$ larger than that to which room temperature was found at 4 °C (data not shown), indicating that the $k_{obs}$ values at the higher temperature and ATP concentrations had not been underestimated due to limitations of the manual quenching procedure.

A minimal reaction scheme for the catalytic cycle of the cross-linked ATPase is shown in Scheme 1. We will show below that the rate constants for dephosphorylation and deocclusion of Ca\textsuperscript{2+} are slow compared with the other rate
The concentration of enzyme is close to that of the nucleotide in the range in which phosphoenzyme levels increase.

The rate of dephosphorylation following addition of EGTA and EGTA plus ADP is shown in Fig. 5. In the absence of ADP, dephosphorylation takes places in a monophasic and first order manner with a rate constant of 0.01 s⁻¹ (Scheme 1, kₘ). Rendering the vesicles leaky with 4% (w/w) A23187 did not alter this value. The same value was obtained in cold chase experiments in which [γ-³²P]ATP was followed with excess unlabeled ATP (results not shown). For these reasons, this step represents a maximum kₘ value for the cross-linked enzyme in the forward direction of catalysis since it is the slowest step in the cycle. Under these conditions and in the presence of 4% (w/w) A23187, the native enzyme hydrolyzed ATP (1 mM) with a turnover time of approximately 10 s⁻¹, which is in the range reported in the literature (20 s⁻¹ at 25 °C) (16). The value obtained with the cross-linked ATPase is therefore approximately 3 orders of magnitude slower than that of the native enzyme.

ADP accelerates dephosphorylation in a monophasic and pseudo-first order manner with kₘ = 0.17 s⁻¹ at saturation. All of the phosphoenzyme was ADP-sensitive. The data fit well to a mechanism of rapid equilibrium of ADP binding (kₑ = 1.8 mM) to the phosphoenzyme followed by first order dephosphorylation. The reaction kₘ (Scheme 1) is insignificant since the off rates of Ca²⁺ (17) and ATP (Kᵦ in the millimolar range) are likely to be fast compared with kₘ. Therefore, kₘ at saturation approximates kₘ. The equilibrium constant for the phosphoryl transfer reaction for the cross-linked enzyme (kₘ/kₖₛ) is then 4.1. The reaction of ADP with the native phosphoenzyme formed in the presence of Ca²⁺ is biphasic and occurs under conditions similar to those used here (16, 18–20). The faster phosphoryl transfer reaction occurs with a rate constant greater than 150 s⁻¹ (16) and the slower reaction, which represents either Eₘ-P conversion to Eₔ-P or ATP release, exhibits a rate constant of 37 s⁻¹ (16). The lack of a burst phase of dephosphorylation on the addition of saturating levels of ADP strongly suggests that it is the phosphoryl transfer step which is slowed by the cross-link, rather than a subsequent step. The reaction is slowed approximately 3 orders of magnitude. The Kₑ,ₐd for the phosphohydrolyzed native enzyme is 0.75 mM (15), which is similar to or slightly lower than that of the cross-linked enzyme.

FIG. 3. Kinetics of MgATP-dependent phosphorylation. In A, phosphorylation of SR preparations cross-linked to varying extents (closed squares, open triangles, and open circles) are compared to control preparations (closed circles and closed triangles). Cross-linking was carried out under standard conditions for 70 min in the absence (open circles, 90% E(125)) or presence of 2 mM triethanolamine sulfate (closed squares, 40% E(125)), for 25 min in the absence (open triangles, 65% E(125)), or presence (closed triangles, <5% E(125)) of 0.12 mM AMPPCP. The other control preparation (closed circles) was incubated for 70 min in the absence of glutaraldehyde. Phosphorylation was performed in 47 mM MOPS/Tris, pH 7.4, 85 mM KCl, 4.2 mM MgCl₂, 0.05 mM CaCl₂, 33 mM sucrose, 0.063 mg of SR protein/ml, and 0.02 mM ATP. In B, the kinetics of ATP concentration dependence of phosphorylation of cross-linked ATPase (E(125) > 95%) is shown. Phosphorylation was performed in the same medium as in A with 0.005 (open circles), 0.02 (closed circles), 0.05 (open triangles), 0.1 (closed triangles), and 1 (open squares) mM [γ-³²P] ATP. The data are fitted to a first order equation. The lines have been constrained to go through 0.13 nmol of E-P/mg of protein. This figure was obtained from the extrapolated zero points for the unweighted best fit curves at 0.005 and 0.02 mM ATP. It represents 3.4% of the maximum phosphoenzyme level and is likely due to the uncross-linked ATPases. This value is not expected to change at the higher ATP concentrations as the kₘ for the native enzyme is 0.066 μM (21). Inset, plot of observed rate constants of phosphorylation (kₘ) versus ATP concentration. Points obtained at 0.2 and 0.5 mM ATP are from data not shown in the main figure. These data sets were omitted for clarity.

\[
\begin{align*}
E + 2Ca^{2+} + ADP & \rightarrow E - P(2Ca) \\
E - P(2Ca) + Ca^{2+} & \rightarrow E - P(2Ca) \\
E - P(2Ca) + Ca^{2+} & \rightarrow E
\end{align*}
\]

The rate of cross-linking following addition of EGTA and EGTA plus ADP is shown in Fig. 5. In the absence of ADP, dephosphorylation takes places in a monophasic and first order manner with a rate constant of 0.01 s⁻¹ (Scheme 1, kₘ). Rendering the vesicles leaky with 4% (w/w) A23187 did not alter this value. The same value was obtained in cold chase experiments in which [γ-³²P]ATP was followed with excess unlabeled ATP (results not shown). For these reasons, this step represents a maximum kₘ value for the cross-linked enzyme in the forward direction of catalysis since it is the slowest step in the cycle. Under these conditions and in the presence of 4% (w/w) A23187, the native enzyme hydrolyzed ATP (1 mM) with a turnover time of approximately 10 s⁻¹, which is in the range reported in the literature (20 s⁻¹ at 25 °C) (16). The value obtained with the cross-linked ATPase is therefore approximately 3 orders of magnitude slower than that of the native enzyme.
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conditions under which both occluded and transported Ca\textsuperscript{2+}
are measured, showing that Ca\textsuperscript{2+} is transported into the vesicle
interior (Fig. 6B). The initial rate of transport at 0.5 mM ATP
is approximately 1 nmol/s/mg of protein. This rate is 10-fold
faster than the \( k_{\text{cat}} \) of the cross-linked enzyme, and evidently
there is a significant contribution to the transported Ca\textsuperscript{2+}
from the small percentage of rapidly turning over uncross-
linked ATPases. The rate of transport by the native ATPase
in the initial phase was measured as 22 nmol Ca\textsuperscript{2+}/s/mg
of protein under these conditions, and a few percent unmodified
ATPase could account for the discrepancy. Note that the
uncross-linked ATPases make a significant contribution to
the transported Ca\textsuperscript{2+}.

An interesting question is whether the inhibition of Ca\textsuperscript{2+}
translocation by the cross-link is partial or complete. It seems
possible that turnover of the cross-linked enzyme could follow
an uncoupled pathway with the direct hydrolysis of \( E_1 \).
particular at ambient temperatures, due to limitations in

with acetyl phosphate compared with ATP was obtained at

poor binding affinity of ADP and acetate and their low
to diminishing the level of phosphoenzyme because of the

of Ca\textsuperscript{2+} occlusion at saturating concentrations of substrate,

phosphate, respectively, probably do not contribute significantly

the highest concentration of acetyl phosphate used in the

ysis is 4-6.5 mM (20, 22) and that for phosphorylation is 43

bound ADP or bound acetate to yield ATP and acetyl phos-

smaller nonnucleotide substrates, such as acetyl phosphate

and p-nitrophenyl phosphate, were relatively rapid (Fig. 8).

at these concentrations of substrate (1 mM) and the

indicates that either the cross-linked enzyme is not saturated

of ATP. Since the rates of deocclusion and hydrolysis are

is expected to be the same, irrespective of the substrate, this

occlusion (in the presence of A23187) by GTP and ITP were

lower than that of ATP or, if saturated, the rate constants

for Ca\textsuperscript{2+} leak from the vesicle lumen.

Other substrates, besides ATP, were tested for their ability
to occlude Ca\textsuperscript{2+}. At a concentration of 1 mM, the rates of

occurrence (in the presence of A23187) by GTP and ITP were

approximately 50- and 100-fold lower, respectively, than that

of ATP. Since the rates of deocclusion and hydrolysis are

expected to be the same, irrespective of the substrate, this

indicates that either the cross-linked enzyme is not saturated

at these concentrations of substrate (1 mM) and the

K_m is higher than that of ATP or, if saturated, the rate constants

of phosphoryl transfer are lower for these substrates compared

with ATP. Alternatively, both mechanisms may contribute.

GTP and ITP have 50-100-fold higher K_m values for phos-

phorylation of the native enzyme than ATP (45 and 31 \mu M,

respectively, compared with 0.066 \mu M; Ref. 21), and it is

conceivable that the cross-link sterically hinders their binding

as much as that of ATP, thereby shifting the K_m values to

higher than 1 mM. In contrast, the rates of occlusion by

smaller nonnucleotide substrates, such as acetyl phosphate

and p-nitrophenyl phosphate, were relatively rapid (Fig. 8).

In fact, higher levels of Ca\textsuperscript{2+} occlusion were obtained with

acetyl phosphate than with ATP (Fig. 8A, compared with Fig.

6A) and could indicate that the rate of phosphorylation is

actually faster with the smaller substrate. The steady state

level of occluded Ca\textsuperscript{2+} is determined principally by the relative

rates of formation and breakdown of the occluded species in the

forward direction of catalysis since the back reactions of

bound ADP or bound acetate to yield ATP and acetyl phos-

phate, respectively, probably do not contribute significantly
to diminishing the level of phosphoenzyme because of the poor

binding affinity of ADP and acetate and their low concentration.

Direct evidence of a faster rate of occlusion with acetyl phosphate compared with ATP was obtained at lower temperatures as explained below.

The K_m of the native enzyme for acetyl phosphate hydroly-
sis is 4-6.5 mM (20, 22) and that for phosphorylation is 43

mM (20), under quite similar conditions. Therefore, even at

the highest concentration of acetyl phosphate used in the

experiments reported in Fig. 8 (1 mM), the concentration was

lower than the K_m. It was not possible to measure the rates of

Ca\textsuperscript{2+} occlusion at saturating concentrations of substrate,

particularly at ambient temperatures, due to limitations in

the manual filtering methodology and to the fact that the reaction could only be stopped with an EGTA solution and hence depended on Ca\textsuperscript{2+} dissociation. In a direct comparison of ATP- and acetyl phosphate-supported Ca\textsuperscript{2+} occlusion at close to 0°C and at concentrations of substrate closer to saturation (1 mM ATP and 30 mM acetyl phosphate), k_{obs} for the smaller substrate was approximately 10-fold faster than that of the nucleotide. This suggests that the cross-link selectively inhibits the nucleotide-dependent process and not that of acetyl phosphate.

A comparison of the initial rates of Ca\textsuperscript{2+} sequestration by control and cross-linked preparations in the absence of A23187 using p-nitrophenyl phosphate as substrate is shown in Fig. 8B. The reason for choosing p-nitrophenyl phosphate is that it is a very poor substrate of the Ca\textsuperscript{2+}-ATPase and phosphorylation and Ca\textsuperscript{2+} occlusion are rate-limiting under optimal turnover conditions. In fact, the level of phosphoenzyme even at saturating levels of substrate is below the limits of detection (23). Also, phosphorylation and Ca\textsuperscript{2+} occlusion are virtually irreversible because of the low affinity of the phosphoenzyme for the product p-nitrophenol and the low concentration of the latter during the reaction. Therefore, the ATPase will turn over in the forward direction of catalysis on cessation of further phosphorylation following addition of the EGTA quench and both occluded and transported Ca\textsuperscript{2+} will be measured. The initial rate of Ca\textsuperscript{2+} sequestration by the cross-linked enzyme was approximately twice that of control preparations. A similar result was obtained using low concentrations of acetyl phosphate as substrate (not shown). If it is assumed that the passive leak rate is not affecting the rate of transport of control vesicles in the initial phase, these results suggest that the rate of phosphorylation and Ca\textsuperscript{2+} occlusion of the cross-linked enzyme is approximately double that of the native enzyme. This conclusion should, however, be treated with some caution, as the extent to which passive efflux does influence the initial rate of transport of control vesicles is difficult to assess. We do know that if the permeability is increased deliberately with A23187, Ca\textsuperscript{2+} sequestration by the cross-linked preparation is not affected (Fig. 8B), but in the case of control vesicles no transport can be measured at all. It seems safe to conclude that if the cross-link does not actually accelerate Ca\textsuperscript{2+} occlusion and phosphorylation, it certainly does not appear to inhibit it.

![Fig. 7. Ca\textsuperscript{2+} release in the presence and absence of A23187 and the effect of ADP. Ca\textsuperscript{2+} uptake was carried out as in Fig. 6A with 25 \mu M ATP, in the presence (closed circles) and absence (open circles) of A23187. At time 0 (arrows), the SR vesicles were perfused with a wash medium (see “Experimental Procedures”) containing either EGTA (1 mM) (circles) or EGTA (1 mM) + ADP (2 mM) (triangles). Ca\textsuperscript{2+} release curves have been fitted by nonlinear regression to a first order process. The curves were constrained to go through the zero time points. The rate constants were 0.012 s\textsuperscript{-1} (closed circles), 0.0083 s\textsuperscript{-1} (open circles), and 0.079 s\textsuperscript{-1} (triangles).](http://www.jbc.org/content/273/11/4618.full`

![Fig. 8. Acetyl phosphate- (A) and p-nitrophenyl phosphate- (B) dependent Ca\textsuperscript{2+} uptake of cross-linked and control ATPase. A, uptake was performed in a medium similar to that in Fig. 6A except that, instead of ATP, acetyl phosphate (AcP) was used at concentrations of 0.02 (open triangles), 0.050 (closed triangles), 0.1 (open circles), and 1 (closed circles) mM. All experiments were carried out with cross-linked ATPase. B, uptake was performed as in Fig. 6A, except, instead of ATP, 1 mM p-nitrophenyl phosphate (p-NPP) was used and the vesicles were either cross-linked (circles) or not (triangles) and the uptake medium contained either 4% (w/w) A231287 (closed circles) or not (open triangles). XL, cross-linked.](http://www.jbc.org/content/273/11/4618.full)
It is relevant to the mechanism of the cross-link induced stabilization of $E_1 \sim P(2Ca)$ that this intermediate is the most reactive of the principal catalytic intermediates to glutaraldehyde, in respect to cross-link formation. We have shown previously that the other main phosphorynome intermediate, $E_1P$, is completely unreactive (6). The enhanced reactivity of $E_1 \sim P(2Ca)$ over the Ca$^{2+}$-bound form is shown in Fig. 9, which compares the amount of $E(125)$ formed before and after induction of native ATPase turnover with acetyl phosphate in the presence of 5 mM Ca$^{2+}$ (2nd through 5th lanes) and in the presence of 5 mM Mg$^{2+}$ and 0.1 mM Ca$^{2+}$ (6th through 9th lanes), both in the presence of high salt concentrations. The latter ensures that the amount of $E_1P$ is low and helps to increase the proportion of $E_1 \sim P(2Ca)$. The rate of cross-link formation is sensitive to pH and care was taken to ensure that the addition of substrate and the reaction did not change the pH by more than 0.05 pH units (100 mM MOPS/triethanolamine buffer). The gels show that the amount of $E(125)$ is increased 2.9–3.5-fold in the presence of substrate. The actual increase in reactivity of the phosphorynome may be slightly more than this since the steady state level of $E_1 \sim P(2Ca)$ is almost certainly less than the amount of Ca$^{2+}$-bound enzyme at equilibrium with which it is compared. Thus, at least three conformations of the active site can be distinguished in respect to their reactivity to glutaraldehyde; the Ca$^{2+}$-free and Ca$^{2+}$-bound forms (at alkaline pH), which are moderately and equally reactive (5); the ADP-sensitive phosphorynome, which is most reactive, as shown above; and the ADP-insensitive phosphorynome, which is unreactive (6).

**DISCUSSION**

Intramolecular cross-linking of the active site of the Ca$^{2+}$-ATPase with glutaraldehyde is shown in this study to affect all of the principal steps of the normal catalytic cycle except for Ca$^{2+}$ binding and the associated changes in tryptophan fluorescence. The most striking effects of the cross-link on catalysis are the large decrease in affinity for ATP, the very slow rate of nucleotide-dependent phosphorylation and Ca$^{2+}$ occlusion and of dephosphorylation and deocclusion, the strong stabilization of the phosphorynome with occluded Ca$^{2+}$, and the inability to form the $E_2P$ intermediate in both directions of catalysis. Ca$^{2+}$ occlusion and, presumably, phosphorylation with smaller substrates are unaffected or even perhaps accelerated. In seeking to explain these effects of the cross-link, it is important to try to distinguish between effects mediated by steric hindrance of ligand binding and those due to interference in the catalytic mechanism. Of particular interest in the latter category, inasmuch as the modification is an intramolecular cross-link, is the possibility of restriction of an essential tertiary structural change at the active site. It has proved valuable to use different sized substrates to distinguish between the different mechanisms of inhibition, as was the case with the fluorescein 5'-isothiocyanate modification (24, 25) and photoaffinity labeling with 2',3'-O-(2,4,6-trinitrophenyl)-8-azido-adenine nucleotides (26). In the former case, the B tryptic fragment is modified at Lys-515 (27), and in the latter case, the A1 fragment is labeled at an as yet unidentified residue. In both cases, ATP binding and phosphorylation are blocked, and yet enzyme turnover with acetyl phosphate or phosphorylation in the reverse direction of P, readily occurs. In the case of the cross-link, which connects tryptic fragments A1 and B, ATP binding is strongly impaired, but obviously not completely, because nucleotide-dependent reactions occur, although at a very slow rate compared with the native enzyme. Phosphorylation per se appears to be unaffected or, perhaps, marginally accelerated when small substrates such as acetyl phosphate are used. The striking difference with the cross-link modification is that subsequent enzyme turnover, involving Ca$^{2+}$ release to the vesicle lumen and conversion to the ADP-insensitive $E_2P$, or phosphorylation by P, in the reverse direction is strongly inhibited. Several considerations suggest that this additional effect is due mainly to the cross-link preventing a conformational change at the active site associated with the formation of low affinity and inward-oriented Ca$^{2+}$ transport sites.

Since the cross-link does not directly inhibit phosphorylation and Ca$^{2+}$ occlusion, the very slow rate of these processes with nucleotides as substrates (ATP, GTP, and ITP) must be due to the strong inhibition of nucleotide binding and their transformation into relatively poor substrates. The maximum rate of phosphorylation with ATP is similar to that exhibited by the native enzyme with p-nitrophenyl phosphate as substrate ($k_{cat} \approx 1$ s$^{-1}$ for p-nitrophenyl phosphate (28) and assuming phosphorylation is rate-limiting). The rates are much slower with GTP and ITP. The cross-link changes the configuration of the active site, with respect to nucleotide binding, such that the normal pattern of high affinity/high activity becomes low affinity/low activity. It is a clear demonstration of the important role of binding energy and the correct positioning of the species directly participating in the chemical reaction in attaining the transition state and rate enhancement as well as in determining the specificity of the reaction. The cross-link differentially affects the binding of ATP to the unphosphorylated ATPase and ADP to the phosphorynome. In the direction of ATP synthesis, the affinity of the native phosphorynome for ADP is low and is hardly changed by the cross-link, and yet the reaction is also slowed by approximately 3 orders of magnitude. According to concepts outlined by Jencks (29, 30), the binding energy associated with the interaction of substrate and enzyme is often not entirely expressed in the binding affinity but rather in altering the configuration of residues at the active site to lower the energy difference between the ground state and the transition state and thereby to enhance the rate of the reaction. The binding energy derived from ADP binding to the native ATPase is presumably expressed in the rate and not in the affinity. With the cross-linked enzyme, the binding energy for ADP could be very much less, with virtually all of it expressed in

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**Fig. 9. Effect of enzyme turnover on the reactivity of the ATPase to glutaraldehyde.** Glutaraldehyde (1.7 mM, 3500-fold dilution of 25% solution) was reacted with SR vesicles (0.5 mg of protein/ml) for 2 min at room temperature in 100 mM MOPS/triethanolamine, pH 7.8, 5 mM CaCl$_2$, 200 mM NaCl with or without acetyl phosphate (AcP) as indicated (5 mM Ca$^{2+}$) or in 100 mM MOPS/triethanolamine, pH 7.8, 5 mM MgCl$_2$, 100 mM NaCl, 0.1 mM CaCl$_2$, and the concentrations of acetyl phosphate indicated (5 mM Mg$^{2+}$ + 0.1 mM Ca$^{2+}$). The first lane contains SR not treated with glutaraldehyde. Gels showed that the increases in $E(125)$ with 2 mM acetyl phosphate as compared with the absence of acetyl phosphate were 2.9- and 3.5-fold in the Ca$^{2+}$ medium and Mg$^{2+}$ + Ca$^{2+}$ medium, respectively.
the affinity rather than in the rate.

In regard to a possible accelerating effect of the cross-link on Ca\(^{2+}\) occlusion and phosphorylation when small substrates are used, it is pertinent that the occluded species, \(E_1 \sim P(2Ca)\), is cross-linked approximately 3-fold more rapidly than \(E_1\), suggesting that the cross-linked residues are in a more favorable position or ionization state to form the cross-link once the active site is phosphorylated. Phosphorylation of the native ATPase with ATP involves initial induction of a conformation that permits extremely rapid phosphoryl transfer to the protein (14). Small substrates, such as acetyl phosphate, presumably do not induce this conformation, and this probably contributes to the slower phosphorylation rate (20). It seems possible that the cross-link, which is clearly located at the ATP-binding site, may mimic ATP binding and stabilize a transition state in the same way ATP must, thereby accelerating phosphorylation by the smaller substrates. This effect may entail pulling the nucleotide-binding domain toward the phosphorylation domain and partially closing the active site (see Fig. 10). The higher reactivity of \(E_1 \sim P(2Ca)\) to glutaraldehyde compared with that of \(E_1\), and hence the relative stabilization of the former, is consistent with this proposal and suggests that the configuration of the active site in the transition state more closely resembles that of the ground state of the phosphoenzyme than the unphosphorylated enzyme.

A striking effect of the cross-link on catalysis is the strong inhibition of \(E_2-P\) formation. In fact, it is possible that the cross-linked ATPase may not be able to attain this state at all. The inhibition is most likely related to the prominent conformational change at the active site that is known to accompany the formation of this intermediate. A thermodynamic analysis of the catalytic cycle of the native enzyme based on experimentally determined equilibrium constants of the partial reactions indicates that the reactions \(E_2 + P_1 \rightarrow E_2-P\) and \(E_1 \sim P(2Ca) \rightarrow E_1-P + 2Ca^{2+}\) correspond to large changes in Gibbs free energy (10.6 and \(-12.5\) kcal/mol, respectively; Ref. 31). Perhaps the most obvious evidence of a significant structural transition at the active site on formation of this intermediate is the large increase in fluorescence of trinitrophenyl nucleotides bound (32-36), or covalently attached (26), at the active site. Another indication of a local conformational change is our previous finding that the \(E_2-P\) intermediate is unable to be cross-linked with glutaraldehyde (6), which is consistent with the inability of the cross-linked enzyme to reach this conformation. We have suggested previously that the active site closes, resulting in an extrusion of water and an increase in hydrophobicity (6). The cross-link may be positioned at a critical hinge region restricting interaction of putative nucleotide and phosphorylation domains.

The finding that the formation of \(E_2-P\) is inhibited in both directions of catalysis suggests that similar active site movements are executed during the loss in ADP sensitivity in the forward direction and upon P\(_i\) phosphorylation in the reverse. It seems logical that these changes are linked to similar structural changes in the region of the Ca\(^{2+}\) sites. If this were the Na\(^{+}\),K\(^{+}\)-ATPase, it would be clear that the steps are involved in deocclusion of cations to the trans or extracellular side of the membrane, Na\(^{+}\) in one direction of catalysis and K\(^{+}\) in the other (37-40). The existence of a counterion to Ca\(^{2+}\) in the Ca\(^{2+}\)-ATPase is controversial; there is evidence to suggest that it may be H\(^{+}\) or possibly K\(^{+}\) (41-43). A requirement for protonation of the ATPase for phosphorylation with P\(_i\) (44) perhaps points to H\(^{+}\) as the counterion. However, even if there is no counterion, the close homology of the Ca\(^{2+}\) - and Na\(^{+}\),K\(^{+}\)-ATPases almost certainly points to the existence of an occluded form of the transport sites in the unphosphorylated Ca\(^{2+}\)-ATPase. Our cross-link results may then be interpreted as a stabilization of a conformation of the active site that is linked to the occluded state of the transport sites, with Ca\(^{2+}\) occluded in the forward direction of catalysis and either vacant or containing counterions in the reverse direction (see Fig. 10). In both cases, the next step, namely mutual destabilization of the occluded cations and the phosphoryl group (bound in one case and covalent in the other) through possibly closing the active site and opening of the distal end of the transport sites, is blocked.

In Fig. 10, we show a possible relationship between the conformations of the active site for the different catalytic intermediates based on their reactivity to glutaraldehyde and the status or orientation of the transport sites. Recent studies suggest that the two Ca\(^{2+}\) ions are translocated sequentially (45, 46) and that the location of the Ca\(^{2+}\) sites is within the membrane-located section (3). Hence, the translocation may simply involve gating on either side of the bound cations. The identification of a distinct intermediate with occluded Ca\(^{2+}\) (47-50), confirmed in this study, suggests that translocation takes place in two gating steps. Ca\(^{2+}\) binding is followed by closure of a "gate" behind the Ca\(^{2+}\) ions with phosphorylation; then another gate is opened to the lumen, accompanied by destabilization of the bound Ca\(^{2+}\) to facilitate its release. In the figure, the active site of the occluded forms has been designated as "ajar" to distinguish them from the postulated "open" and "closed" forms of \(E_1\) and \(E_2-P\), respectively; the distinctions are based on the forms' relative reactivity to glutaraldehyde and are indicated on the right-hand side of the catalytic scheme. The occluded form of the pump in the unphosphorylated state has been left vacant but, as discussed above, it could be occupied by H\(^{+}\) or K\(^{+}\). The model suggests that local tertiary structural changes at the active site, essentially a two-step cleft closure around the phosphoryl group, trigger specific changes in the Ca\(^{2+}\)-binding region to gate and destabilize the transport cations. Energy transduction is achieved through the shifting of linking helices, similar to those known to occur in allosteric soluble enzymes (4).

There are other modifications of the ATPase that also selectively inhibit steps 3 and 4. N-Ethylmaleimide labeling of Cys-344 and Cys-364 partially inhibits both steps (36, 51). Derivatization with dicyclohexycarbodiimide, under mild
conditions that may also result in intramolecular cross-linking, selectively blocks ATPase activity and P_i phosphorylation, without affecting the level of phosphoprotein formed from ATP (52). Mutation of conserved residue Gly-233 inhibits Ca^{2+} release and E_2-P formation in both directions of catalysis, although the inhibition of P_i phosphorylation in the reverse direction may be overcome with high concentrations of P_i (53). On the other hand, several modifications result in catalysis, although the inhibition of P_i phosphorylation from Ca^{2+} release and E_2-P formation in both directions of catalysis. Mutation of conserved residue Pro-312 (54) and modification of Cys-344 with 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole (55) fall into this category, and evidently the similarities between steps 3 and 4 cannot be taken too far.

In conclusion, the block to E_2-P caused by the cross-link may be rationalized in terms of obligatory conformational mechano-coupling in which the different steps of the catalytic cycle are accompanied by conformational changes at the active site that are linked, in a mechanical fashion, to the membrane region of the protein to alternate the opening and closing of gates at either end of the two Ca^{2+} transport sites and destabilize the occluded cations. Cross-linking reagents that prevent these movements and hence block the transduction of energy through the protein can be expected to inhibit catalysis and transport. The high stability that the cross-link confers on the E_2 ~ P(2Ca) intermediate with occluded Ca^{2+} and on the E_2 intermediate, possibly with occluded H^+ or K^+, will hopefully facilitate further investigation of the gating mechanisms.

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