Intramolecular Cross-linking at the Active Site of the Ca\(^{2+}\)-ATPase of Sarcoplasmic Reticulum

HIGH AND LOW AFFINITY NUCLEOTIDE BINDING AND EVIDENCE OF ACTIVE SITE CLOSURE IN E\(_1\)-P\(^*\)

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Limited reaction of glutaraldehyde with the Ca\(^{2+}\)-ATPase (\(M \approx 110,000\)) of sarcoplasmic reticulum results in intramolecular cross-linking at the active site, which can be detected by an anomalous increase in apparent molecular weight (\(M \approx 125,000\)) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Ross D. C., and McIntosh D. B. (1987) J. Biol. Chem. 262, 2042–2049). ATP, ADP, AMPPCP, trinitrophenyladenosine triphosphate, and decavanadate inhibited the cross-link in a manner suggestive of a homogeneous class of inhibitory sites, with \(K_{i,0}\) values for inhibition in agreement with \(K_i\) values for binding to the active site. Cross-link formation was inhibited in proportion to phosphoenzyme levels formed from Pi (E\(_2\)-P) had no effect. Inhibition was observed at millimolar concentrations of CaATP, indicative of nucleotide-binding domains, respectively. Location of the cross-link in the micromolar and millimolar concentration ranges, the former attributable to E\(_1\)-ATP and E\(_2\)-P formation and the latter to ATP binding mainly to E\(_1\)-P. The inability to cross-link the active site only of the E\(_2\)-P intermediate suggests a unique active site conformation, possibly a closed active site cleft, which we suggest is linked to low affinity, inwardly orientated Ca\(^{2+}\)-binding sites.

The active site of the Ca\(^{2+}\)-ATPase of rabbit skeletal muscle sarcoplasmic reticulum (SR)\(^1\) has recently been proposed to consist of segments of a nucleotide-binding domain and a phosphorylation domain (1). Structural analogies with phosphoglycerate kinase and hexokinase further suggest that a 60-residue section of \(\alpha\)-helices may form the hinge between the two domains making up the active site. However, there is no evidence, as yet, that any of the conformational changes which are known to occur at the active site are linked to interdomain hinge bending and active site closure. Ca\(^{2+}\) binding to the high affinity transport sites switches the aspartyl residue in the phosphorylation domain from Pi-reactive (E\(_1\)) to ATP-reactive (E\(_2\)-2Ca) (Scheme 1, Refs. 2–4). ATP binding in the presence of Ca\(^{2+}\) triggers a conformational change which may, as a result of an interdomain movement, appose the aspartyl residue with the \(\gamma\)-phosphoryl group of ATP bound to the other domain (5). Further rearrangements at the active site can be expected when the phospho-aspartyl group changes from ADP-reactive (E\(_1\)-P) to ADP-unreactive (E\(_2\)-P) in a subsequent step (6, 7). This latter change is accompanied by translocation of Ca\(^{2+}\) from the high affinity to the low affinity sites (8, 9). In addition, less well-defined intermediates may reflect other movements.

Another outstanding issue is the nature of ATP regulation of catalysis by the Ca\(^{2+}\)-ATPase. ATP, at concentrations in excess of that required to saturate the active site, accelerates the E\(_1\) to E\(_2\)-Ca transition (10–12), the conversion of E\(_1\)-P to E\(_2\)-P (13, 14), and dephosphorylation (7, 14). These effects together are responsible for the well-known increase in rate of ATP hydrolysis and Ca\(^{2+}\) transport in the millimolar ATP concentration range (15–23). The nature of the effector site(s) is unclear. A separate allosteric site (19–25) or an altered form of the catalytic site, arising either as a result of subunit interactions (20, 21), or the generation of catalytic intermediates (14, 19, 23), have all been considered. In the latter case, ATP may bind with low affinity to the active site following ADP departure in the E\(_1\)-P state. Choosing between the models is difficult because of uncertainties regarding the concentration of active sites, problems in measuring low affinity ATP binding, and of distinguishing between nucleotide binding at the active site, and elsewhere on the protein.

We have reported that glutaraldehyde reacts with the Ca\(^{2+}\)-ATPase to form an intramolecular cross-link at the active site, which can be detected by SDS-PAGE because of its retarded mobility (26). The cross-link was shown to connect tryptic fragments A and B, which constitute the proposed phosphorylation and nucleotide-binding domains, respectively. Location of the cross-link at the active site, and the fact that it appears to span the two domains, makes it a potentially useful probe of events at the active site, of active

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The abbreviations used are: SR, sarcoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)-propanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; EGTA, ethylenbis(oxyethylenenitriilo)tetrasacetic acid; E\(_1\)(25), enzyme with \(M \approx 120,000\); AMPPCP, adenosine 5'-[\(\alpha,\beta\)-methylene]triphosphate; TNP-ATP, trinitrophenyladenosine triphosphate.
site conformation, and possibly, of interdomain movement. We show here that cross-link formation is inhibited by nucleotide occupancy of the active site and by formation of the ADP-insensitive phosphoenzyme (E\(_{125}\)-P) but not by formation of the ADP-sensitive phosphoenzyme (E\(_{110}\)-P), nor by any other nucleotide-devoid intermediate. Inhibition patterns during catalysis indicate that ATP binds with low affinity to E\(_{110}\)-P.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutaraldehyde (Grade II, 25% aqueous solution), hydrazine, AMPPCP and Triton X-100 were purchased from Sigma. Triton X-100 was treated to remove oxidizing contaminants (27).

SR vesicles were prepared from rabbit back and hind limb white muscle (28). They were stored as a suspension (10–20 mg of protein/ml) in 10 mM imidazole, pH 7.4, and 0.3 M sucrose at –60 °C. Protein concentrations were estimated by the Lowry method, using bovine serum albumin as standard and sodium deoxycholate as a solubilizing agent.

_Cross-linking_—SR vesicles (0.4 mg of protein/ml) were reacted with 5 mM glutaraldehyde for 4 min, unless otherwise specified, at 25 °C in a medium indicated in the legends to the figures. The reaction was terminated by a 5–10-fold molar excess of hydrazine.

_Gel Electrophoresis_—This was carried out according to Laemmli (29) as described before (30). Coomassie Blue stained and dried gels were scanned with a Vitatron TLD.

**ATPase Activity**—ATP hydrolysis catalyzed by SR vesicles, made permeable to Ca\(^{2+}\) with A23187, was determined from the rate of \([\gamma^{32}P]ATP\) release from \([\gamma^{32}P]ATP\) using the molybdic acid procedure (31). \([\gamma^{32}P]ATP\) was synthesized as in Ref. 32.

**Phosphoenzyme**—Phosphorylation from \([\gamma^{32}P]ATP\) and from \([\alpha^{32}P]ATP\) was measured by filtration on a glass fiber filter after acid quenching (33).

**RESULTS**

The Ca\(^{2+}\)-ATPase (\(M_0 \approx 110,000\)) makes up approximately 90% of the total vesicle protein according to SDS-PAGE (Fig. 1, lane 6). Brief incubation of the vesicles with glutaraldehyde (5 mM) resulted in the partial conversion of the native ATPase (E(110)) to a species of higher molecular weight (E(125), lane 1). This has been demonstrated to be due to an intramolecular cross-link, and change in the hydrodynamic properties of the polypeptide in SDS (26). We have also previously shown that ATP and other nucleotides inhibit E(125) formation. Here we show that TNP-ATP inhibited E(125) formation at low concentrations (lanes 2–5). Almost complete inhibition occurred at concentrations equivalent to the concentration of ATPase polypeptides (2.0–3.3 μM) suggesting very tight binding to a single inhibitory site per ATPase. The lower limit of the range of concentration of ATPase polypeptides is derived from the maximum level of phosphoenzyme obtained from P, in the presence of dimethyl sulfoxide (5 mmol/mg of protein, see below), and the upper limit is based on the assumption that all ATPases are active and that they constitute 90% of the total protein (8.2 nmol/mg of protein).

A simple mechanism for the glutaraldehyde reaction and inhibition by nucleotides is indicated in Scheme 2. E(110) is assumed to exist in rapid equilibrium with nucleotide-bound enzyme and glutaraldehyde to react exclusively with the former species to form E(125), with a pseudo first order rate constant, k. To test this mechanism, the time dependence of E(125) formation was measured in the absence of ATP, and in the presence of sufficient ATP (10 μM) to substantially inhibit cross-linkage (Fig. 2). There is a close fit of the experimental data and curves generated by simulation of the above mechanism. This suggests that the initial rate of formation of E(125) is proportional to the concentration of unliganded enzyme at time zero, and inhibition curves can be related directly to nucleotide binding.

The concentration dependence of TNP-ATP, ATP, ADP, and AMPPCP inhibition of the initial rate of E(125) formation, in the presence of Mg\(^{2+}\) (1.5 mM) and EGTA (1 mM), are shown in Fig. 3. Total nucleotide is plotted on the x axis, and the steep curve obtained from TNP-ATP is due to a combination of tight binding and relatively high concentration of enzyme. The data points for each nucleotide fit well to a model of homogeneous and independent nucleotide-binding sites. K\(_s\) values for inhibition obtained under different conditions for each nucleotide are listed in Table I. Ca\(^{2+}\) and Mg\(^{2+}\) by themselves have no effect on cross-linkage. KCl partially inhibited cross-linkage in a concentration-dependent manner, and appropriate controls were necessary. Alongside these values are published K\(_s\) values obtained by other meth-
The value of may explain the discrepancy between our value of and decrease the extent of cross-linkage (see below). This of Mg²⁺ may be complicated by the presence of adenylate kinase in the preparation. This could have a particularly marked effect in the presence of Ca²⁺, as the generation of ADP binding in the presence of adenylate kinase inhibitor, P', P₅-di-(adenosine-5')pentaphosphate (15 µM), had little effect.

The inhibition studies indicate that the different effects of Mg²⁺ and Ca²⁺ on the affinity of the enzyme for nucleotide depends on the identity of the nucleotide. Mg²⁺ enhanced the affinity for ATP but decreased that for AMPPCP and ADP. The presence of micromolar concentrations of Ca²⁺, which converts the enzyme into the E₁ conformation, had little effect on the affinity for AMPPCP. The CaATP and CaADP complexes have significantly lower Kₑ values, compared with their Mg counterparts. Under the conditions used, notably in the absence of KCl, the level of phosphoenzyme obtained in the presence of CaATP is low (<0.5 nmol/mg of protein, see below) and 2Ca-E-CaATP is the predominant species with ATP. KCl moderately decreases the affinity for all nucleotides. The binding of CaATP cannot be measured in the presence of KCl as substantial phosphorylation occurs.

In the presence of dimethyl sulfoxide, P₅ inhibited cross-linkage (Fig. 4). Ca²⁺ blocked this effect. The P₅ inhibition is dependent on the presence of dimethyl sulfoxide. In the absence of this solvent, but under otherwise similar conditions to those of Fig. 4, little or no change in the degree of cross-linking was observed (result not shown).

| Nucleotides and additions | Kₑ values (µM) |
|--------------------------|----------------|
| ATP                      | 8              |
| ATP + Mg²⁺               | 2              |
| ATP + Mg²⁺ + KCl         | 4              |
| ATP + Ca²⁺ (1 mM)        | 0.5            |
| AMPPCP                   | 8              |
| AMPPCP + Mg²⁺            | 48             |
| AMPPCP + Mg²⁺ + Ca²⁺     | 35             |
| ADP                      | 10             |
| ADP + Mg²⁺               | 12-20          |
| ADP + Mg²⁺ + Ca²⁺        | 60             |
| ADP + Ca²⁺               | 7              |
| ADP + Ca²⁺ (1 mM)        | 0.3            |
| ADP + KCl                | 11             |
| TNP-ATP                  | 3.1            |
| TNP-ATP + Mg²⁺           | 0.05           |
| TNP-ATP + Mg²⁺ + KCl     | 0.2            |

The concentration dependence of inhibition of E₁₁₅ by P₅ monovanadate and decavanadate, as well as the concentration dependence of phosphoprotein formation by P₅, is shown in Fig. 5. There is a correlation between phosphoenzyme formation (Kₑ = 0.2 µM) and E₁₁₅ inhibition (Kₑ = 0.38 mM). Monovanadate provided partial inhibition at high concentrations. The curve is shallow and not indicative of hyperbolic binding. Binding of monovanadate at the P₅ site occurs at much lower concentrations, with Kₑ values in the range 1-50 µM (pH 7-8) in the presence of Mg²⁺ (44-46). Evidently, complexation of monovanadate at the active site is not inhibitory, and this would be in accord with P₅ exerting no effect in the absence of dimethyl sulfoxide. However, the latter results could also be explained by P₅, not binding to the enzyme under these conditions. In contrast, a steep inhibition curve, similar to that obtained with TNP-ATP, was obtained with decavanadate (Kₑ = 0.2 µM). Other studies have also indicated that decavanadate binds tightly (47, 48). The partial inhibition by millimolar monovanadate solutions is likely due to the formation of irreversible complexation of decavanadate with the active site, leading to a decreased affinity for nucleotide binding.
to contaminating polyvanadate species (48).

The effect of CaATP on E(125) formation and the level of phosphoenzyme obtained under similar conditions in the absence of added Mg$^{2+}$ and absence or presence of NaCl is shown in Fig. 6A. In the absence of NaCl, CaATP inhibited E(125) formation at low concentrations and over a narrow range. Under these conditions, the level of phosphoenzyme at 10 μM ATP was 0.2 nmol/mg of protein or approximately 4% of sites. Hence inhibition is due almost entirely to CaATP binding ($K_{0.5} = 0.5 \mu M$) to the active site. Ca$^{2+}$-stimulated ATPase activity was measured as 0.11 μmol/min/mg of protein at 50 μM ATP, under these conditions, and was 27-fold slower than that measured in the presence of 1 mM MgCl$_2$. Inclusion of 200 μM NaCl in the medium (containing Ca$^{2+}$ and not Mg$^{2+}$) resulted in stoichiometric phosphorylation of the ATPase at low concentrations of nucleotide. The steep phosphorylation curve is indicative of a low $K_a$ ($\sim 0.1 \mu M$). The presence of NaCl at this concentration inhibited cross-linkage approximately 70%. There is no inhibition of E(125) formation in the nucleotide concentration range, 0.1–10 μM, in which the enzyme is phosphorylated. E(125) formation is inhibited only in the higher nucleotide concentration range ($K_{0.5} = 0.3 \mu M$). Since almost all ATPases are in the $E_1$-P form under these conditions, the inhibition indicates ATP binding to this species. NaCl was found to have no effect on
ATPase activity. However, an increase in ATP concentration from 50 to 1000 μM increased ATPase activity from 18 to 114 nmol of Pi released/min/mg of protein, under these conditions. In a less comprehensive series of experiments similar results were obtained with KCl.

The effect of ATP concentration on E(125) formation and phosphoenzyme levels in the presence of Mg2+ and Ca2+, and with, or without 150 mM KCl is shown in Fig. 6B. The relevant portion of the gel from which the inhibition data are derived is shown in Fig. 7. In the absence of KCl, increasing MgATP concentrations inhibited E(125) formation in a biphasic manner. Partial inhibition, to the extent of approximately 70%, occurred in the 0.5-5 μM range and further inhibition was apparent in the 0.03-1 mM range. High ATP concentrations resulted in almost complete inhibition of the cross-link. Phosphoenzyme levels were 2.7 nmol/mg of protein at 50 μM ATP and increased to 4.8 nmol/mg of protein at 5 mM ATP. In the presence of 150 mM KCl, the overall level of E(125) is lowered 65%. Inhibition of E(125) occurred only in the millimolar ATP concentration range (Kd = 1.5 mM). The Kd value of MgATP inhibition in the presence of micromolar Ca2+ concentrations (rapid turnover) is hence 5-fold higher than that for CaATP (no Mg2+). Phosphoenzyme levels increased from 2.7 nmol/mg of protein at 50 μM ATP to 3.7 nmol/mg of protein at 5 mM ATP.

The presence of 0.2 or 1.0% (w/w) Triton X-100 had little effect on the biphasic ATP dependence of E(125) inhibition obtained in the absence of NaCl (results not shown). We have shown previously that at the higher detergent concentration the ATPases are predominately monomeric (30).

AMPPCP accelerates ATP hydrolysis up to 2-fold when the concentration of AMPPCP is 20-30-fold higher than that of ATP (23). Under the conditions described in Fig. 6B, but in the presence of 30 mM KCl, 50 μM ATP inhibited cross-linkage approximately 50%. Addition of 1 mM AMPPCP increased the extent of inhibition to approximately 90%. The same effect was observed with 2 μM TNP-ATP. TNP-nucleotides, at low concentrations, also accelerate ATP hydrolysis (25) and become highly fluorescent (49-51). We determined that under the conditions used here 2 μM TNP-ATP accelerated ATP hydrolysis 1.5-fold.

**DISCUSSION**

We have presented evidence previously that the E(125) species represents ATPase which has been cross-linked intramolecularly and as a consequence exhibits anomalous hydrodynamic properties (26). We show there that occupation of the active site by ATP, ADP, and the analogues AMPPCP and TNP-ATP, inhibits cross-linkage in a manner which suggests that the relative proportion of E(110) and E(125) is a measure of nucleotide binding to the active site. The results provide evidence, along with recent fluorescein isothiocyanate modification (23, 40, 52, 53), and direct binding (25, 36) studies, that the ATPases are homogeneous with respect to nucleotide binding under equilibrium or non-turnover conditions. The tight binding inhibitors TNP-ATP and decavanadate suggest that 1 mol of inhibitor blocks 1 mol of active sites and the enzyme exhibits full-sites binding behavior. The potent inhibition by decavanadate is in accord with recent studies indicating tight binding of the polyanion at the active site (47-48).

High concentrations of KCl or NaCl partially inhibited the cross-link. K+ and Na+ are known to modulate catalysis (54-57). Their effect on the cross-link may indicate that they exert their modulations by binding to the active site.

Pn, in the absence of dimethyl sulfoxide, and monovanadate do not inhibit cross-linkage. Also, stoichiometric phosphorylation of the enzyme with CaATP at high KCl or NaCl concentrations, conditions which favor E1-P (57), had no effect. Evidently, the cross-link is not sterically hindered by the phosphoryl group on the aspartyl residue, nor by monovanadate at the active site, and probably neither by Pn.

Phosphorylation to E1-P with Pn, in the presence of dimethyl sulfoxide, inhibited the cross-link, suggesting a difference in active site conformation of this phosphorylated intermediate compared with E1-P, as well as possibly with all the other intermediates of the cycle, at least with those which do not contain bound nucleotide at the active site. This could indicate that either glutaraldehyde has restricted access to the active site, the intersite distance of the reactive residues has changed, or at least one of the reactive residues has become chemically unreactive. Although we cannot distinguish between these mechanisms at present, an attractive possibility is that the active site closes in this conformation. The structure of the active site has been likened to those of hexokinase and phosphoglycerate kinase which are typical hinge-bending enzymes exhibiting ligand-induced active site closure (1, 58). Restricted access of glutaraldehyde could arise by an interdomain movement which closes the active site cleft. This would be in line with evidence of restricted access of water to the active site of the E1-P intermediate (49-51, 59). A corollary to this interpretation is that the active site of E1-P (and other intermediates which readily cross-link) exists in an open conformation. The transition from E1-P to E2-P is a crucial step in the catalytic cycle and kinetic studies have indicated that Ca2+ translocation takes place at this stage (Scheme 1, Refs. 13, 60). The interdomain movement, which accompanies closure of the cleft, may be transduced to the Ca2+-binding domain to effect ion transfer and affinity changes.

In the presence of CaATP and KCl or NaCl, conditions in which phosphoenzyme hydrolysis is low and the enzyme is predominantly in E1-P (13), inhibition at high ATP concentrations is clearly due to nucleotide binding with low affinity to this intermediate. In the presence of Mg2+, which induces rapid enzyme turnover, and high KCl or NaCl, the principal intermediate is also E1-P (54-57, 60-62), and most of the inhibition at high ATP concentrations can again be explained by ATP binding to this intermediate. A small proportion of the inhibition is likely due to ATP binding to E2-P. Binding of ATP to the latter intermediate in this concentration range has been shown from ATP modulations of Pn = HOH oxygen exchange characteristics (14). In the absence of KCl and NaCl, the ATP concentration dependence of inhibition was biphasic. This can be explained by ascribing the inhibition in the low ATP concentration range to increasing steady state levels of E1-ATP and E2-P. In the 5-20 μM range, the inhi-
bition reaches approximately 70% and this can be compared to the 80% ADP-sensitive EP measured by Wakabayashi et al. (Ref. 13, pH 7.0, 5 mM Mg++, 0 °C) and the 41% obtained by Wang (Ref. 61, pH 6.8, 3 mM Mg++, 21 °C). The secondary inhibition at higher ATP concentrations is again explained by ATP binding to principally E1-P.

A low affinity ATP-binding site on the Ca++-ATPase has been inferred for a long time because of the characteristic dependence on ATP concentration, Mg++ and NaCl of the affinity of secondary site for ATP, which is strongly dependent on M+ (13), and inhibition of the former blocks the cross-link. Another possibility is that the secondary site is known to shift the equilibrium of the phos

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