Sarcoplasmic reticulum CaATPase: product inhibition suggests an allosteric site for ATP activation

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Sarcoplasmic reticulum CaATPase hydrolysis of high concentrations of ATP was studied in the presence of ADP. The results obtained were best described as noncompetitive inhibition; added product lowered the V_{max} but did not affect the slopes of Eadie-Hofstee plots. At these concentrations (0.5–5 mM), ATP is known to act as both a substrate and as an activator of turnover. The inability of ATP to overcome ADP inhibition suggests that activating ATP binds to an allosteric regulatory site rather than to the phosphorylated active site.

Sarcoplasmic reticulum *CaATPase* *Product inhibition* *Biphasic kinetics* *Allosteric site*

1. INTRODUCTION

Sarcoplasmic reticulum membranes, as the principal reservoir of Ca^{2+} in skeletal myocytes, acquire this ion in their lumen through active transport by an intrinsic protein, the CaATPase. This enzyme, like many others involved in active transport, shows biphasic kinetics when the velocity is measured as a function of ATP concentration. At substrate concentrations saturating a high affinity K_m in the micromolar regime, the maximum level of E-P (4–5 nmol·mg^{-1}) is formed [1,2]. The further increase in enzyme activity noted at higher ATP concentrations exhibits a dissociation constant, K_r, of several hundred micromolar. This catalytic enhancement can be produced with the nonhydrolyzeable substrate analogue, AMP-PCP [3], which suggests that the low affinity binding of enzyme with activating substrate does not involve hydrolysis. Recently, the amount of CaATPase in the SR membrane was shown to be approximately 5 nmol·mg^{-1} [4]. The close agreement between total enzyme and the maximal amount of E-P formed during steady-state turnover indicates that activating ATP must accelerate the rate limiting step(s) by binding to a phosphoenzyme form rather than noncovalent intermediates or via a half-the-sites mechanism.

Two of the simple models for activating ATP binding are: (1) ATP binds to the phosphorylated active site after the product, ADP, leaves or (2) ATP binds to a regulatory (non-catalytic) site which is either present on all forms of the enzyme or is generated during catalysis. In order to address this question, we have studied the effect of added ADP on the high substrate concentration regime of CaATPase kinetics.

Abbreviations: SR, sarcoplasmic reticulum; E-P, phosphorylated enzyme intermediate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; A_p5A, diadenosine pentaphosphate

2. MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit hind leg white skeletal muscle by the method of Eletr and Inesi [5]. Na-ATP, Tris-ATP,

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cyclohexylammonium-ADP and Tris-*p*-nitrophenyl phosphate were obtained from Sigma. All other chemicals used were reagent grade or better.

For determination of the biphasic ATPase kinetics of the uninhibited enzyme, a coupled enzyme assay was used [6]. The conditions were: 37°C, 80 mM KCl, 5 mM $MgCl_2$, 50 mM Mops (pH 7.0), 1.1 mM $CaCl_2$, 1 mM EGTA, 10 μM Ap5A with 10 μg/ml SR and 0.4 μg/ml ionophore A23187.

The binding constant of ADP to the unphosphorylated enzyme, K_{il}, was determined from the competitive inhibition of ADP (50 μm and 100 μm) seen with CaATPase hydrolysis of *p*-nitrophenyl phosphate. The conditions for measuring *p*-nitrophenyl phosphatase activity were: 37°C, 10 mM $MgCl_2$, 80 mM KCl, 50 mM Mops (pH 7.0), 1.1 mM $CaCl_2$ and 1 mM EGTA. The SR concentration was 50 μg/ml with 2.0 μg/ml ionophore A23187; initial velocities were measured spectrophotometrically at 400 nm.

The activity of the CaATPase in the presence of ADP was determined using the phosphomolybdate assay with the same experimental conditions as for the biphasic kinetics. Each initial velocity was determined from 5 time points in which an aliquot of the reaction mixture was quenched with an equal volume containing 5.8 mM $(NH_4)_6Mo_7O_{24}$ and 168 mM H_2SO_4. The absorbance of each set of 5 time points was then measured at 350 nm. The linear standard curves of A_{350} vs $[P_i]$ determined under the various reaction conditions revealed that the molar extinction coefficient was attenuated by high concentrations of ATP or ADP. The effect of nucleotide concentration on $\Delta\epsilon_{350}$ was small up to 3 mM at which point further increases in [ATP] and [ADP] significantly lowered the extinction coefficient (at zero nucleotide, $\Delta\epsilon_{350}$ = 3000 $M^{-1}\cdot cm^{-1}$; with 10 mM total nucleotides, $\Delta\epsilon_{350}$ = 700 $M^{-1}\cdot cm^{-1}$).

Kinetic constants were estimated with a computer program which uses a nonlinear regression algorithm [14] to fit the data to the following equation:

$$\mathfrak{N} = [V_1S/K_m + V_2S^2/(K_mK_r)]/ [1 + S/K_m + S^2/(K_mK_r) + SI/(K_mK_{i2}) + I/K_{i1} + S/K_r + SI/(K_rK_{il}) + S^2I/(K_mK_rK_{i2})] \quad (1)$$

where v is the velocity; S and I are the concentrations of ATP and ADP, respectively; V_2 and V_1 are the maximal velocities associated with the low and high affinity substrate effects, respectively; K_r and K_m are the corresponding Michaelis constants; and K_{i1} and K_{i2} are the inhibition constants competitive with respect to K_m and noncompetitive with respect to K_r, respectively. The overall goodness of fit is reported as the standard deviation (SD) of the velocities; uncertainties of the parameter values are standard errors of the estimate.

3. RESULTS AND DISCUSSION

Analysis of uninhibited SR CaATPase kinetics using equation (1) resulted in a reasonable fit (SD = 0.3 μmol·min^{-1}·mg^{-1}) which yielded values for a high affinity K_m of 9.5 ± 1.3 μM associated with a lower V_{max}, V_1, of 6.3 ± 0.5 μmol·min^{-1}·mg^{-1} and a K_r of 385 ± 48 μM associated with an upper V_{max}, V_2, of 18.0 ± 0.2 μmol·min^{-1}·mg^{-1} (not shown). Using these

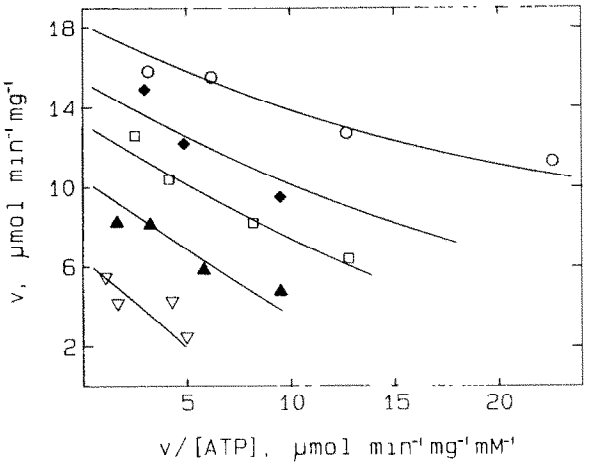


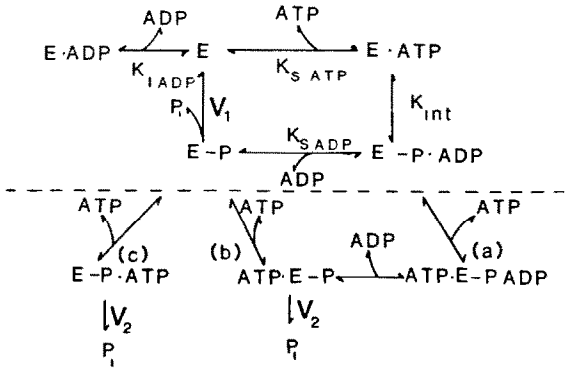
Fig.1. Inhibition of CaATPase activity by added ADP. Velocities were determined using the phosphomolybdate assay as described in section 2. Both nucleotides were added as their Mg complexes; ATP concentration was varied from 0.5 to 5.0 mM. The data were fitted (SD = 0.6 μmol·min^{-1}·mg^{-1}) to eqn 1 as described in section 3, yielding values of: V_2 = 18.2 ± 0.7 μmol·min^{-1}·mg^{-1}, K_r = 0.77 ± 0.17 mM and K_{i2} = 2.7 ± 0.2 mM. The lines were generated using these results. The added concentrations of ADP were: ○, zero; ◆, 0.5 mM; □, 1 mM; ▲, 2 mM; and ▽, 5 mM.

constants for the high affinity regime, the effect of added ADP on V_2 and K_r were analyzed using equation (1). As can be seen in fig.1, added ADP reduces the velocity of ATP hydrolysis so that V_2 (V-axis intercept) is lowered in the presence of product. The data are well described by a process in which added ADP does not change the value of K_r, so that a series of almost parallel curves are obtained. This can be termed noncompetitive inhibition, and is consistent with ATP exhibiting its accelerating effect on rate limiting step(s) which occur after the inhibition by ADP.

These results are discussed in the context of scheme 1 (borrowed with modification from Pickart and Jencks [7]). The mechanism above the dashed line in scheme 1 represents the minimal steps necessary to describe the interactions of ATP and ADP within the turnover of the CaATPase. It can be seen that ADP competes with ATP for the active site of the free enzyme with a dissociation constant, K_{i1}, determined here to be 50 μM. The ratio of the rate of enzyme phosphorylation by ATP to the back reaction rate (enzyme dephosphorylation by ADP) is termed K_{int}. This is followed by dissociation of ADP from the phosphorylated active site, K_{sADP}, and finally, phosphoenzyme hydrolysis.

The rate limiting step(s) of this mechanism which are reflected in V_1 have been postulated to involve calcium release into the lumen which is then followed by rapid hydrolysis of the calcium-free phosphoenzyme [7,8].

The present study addresses the question of how activating ATP accelerates the rate limiting step(s) producing V_2. The possible interactions of activating ATP within the mechanism are shown below the dashed line in scheme 1. Intermediate (c) is a model of ATP binding to the phosphorylated active site; whereas, intermediates (a) and (b) are models of ATP binding to a separate regulatory site. The results shown in fig.1 argue that activating ATP binds in a separate regulatory site, i.e., (c) does not exist. This follows from the observed lack of competition between ADP and activating ATP. If (c) were to exist in rapid equilibrium with E-P·ADP, one would expect ATP to compete with ADP for E-P. The above argument assumes that all phosphoenzyme forms containing bound calcium are in rapid equilibrium relative to turnover. The values of the rate constants associated with K_{int} and K_{sADP} indicate that this is the case. (Pickart and Jencks [7] list values for the rate constants of (a) phosphorylation by ATP of $\geqslant 116\ s^{-1}$, (b) dephosphorylation by ADP of $\geqslant 247\ s^{-1}$, (c) ADP dissociation from E-P of $>3700\ s^{-1}$ and (d) ADP binding to E-P of $5 \times 10^6\ M^{-1} \cdot s^{-1}$. These are large compared to a turnover number of $17\ s^{-1}$.)



Scheme 1

A complication in discerning whether there is a separate regulatory site from product inhibition studies is the possibility that the rate limiting step involves an ADP-insensitive phosphoenzyme not in rapid equilibrium with those forms which can be dephosphorylated by the product. The previously mentioned E-P possessing no bound calcium is ADP-insensitive [9], but occurs after the rate limiting step. If this enzyme form were to be preceded by a calcium containing ADP-insensitive phosphoenzyme whose decomposition were rate limiting, then activating ATP could bind to the active site. If this intermediate were to exist it could not be in rapid equilibrium with ADP-sensitive forms of phosphoenzyme; otherwise, ATP would have competed with ADP and not produced the results seen in fig.1. Such an intermediate, formed in a step which is not readily reversible and decomposed in the rate limiting step, would be expected to build up a large population. However, measurements of ADP-insensitive phosphoenzyme under normal conditions containing high KCl and $MgCl_2$ indicate that it is present as a small fraction of total E-P [10,11]. In fact, Pickart and Jencks [12] studied the effect of ADP on calcium-bound phosphorylated ATPase and found that virtually all of the E-P reacts with ADP, producing kinetics

which indicated all phosphoenzyme forms were in rapid equilibrium with ADP-sensitive E-P. On the other hand, Froehlich and Heller [13] recently inferred the presence of high concentrations of ADP-insensitive E-P during turnover under normal conditions. This conclusion followed from the authors' assigning the slow phase of E-P disappearance in the presence of added product to forms which do not react with ADP. However, the amount of P_i produced in these experiments (which would be produced by ADP-insensitive E-P) was small (17% of total E-P). (When the effect of ADP on V_2 and K_r was studied with no KCl and 0.1 mM free $MgCl_2$ (conditions which favor a large population of ADP-insensitive E-P [10]), we obtained an inhibition pattern which was similar to that shown in fig.1 (not shown).)

Although the present state of knowledge concerning the mechanism of the CaATPase allows for other possibilities, most of the existing information supports a model which has all phosphoenzyme intermediates formed prior to turnover in rapid equilibrium with each other. Assuming this is the case, the present study suggests that ATP exhibits its accelerating effect by binding to an allosteric site.

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