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Effect of pH on the activity of the $Ca^{2+} + Mg^{2+}$-activated ATPase of sarcoplasmic reticulum

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A kinetic model for the $Ca^{2+} + Mg^{2+}$-activated ATPase of sarcoplasmic reticulum was presented in a previous paper [Stefanova, Napier, East & Lee (1987) Biochem. J. **245**, 723–730]. Here, that model is modified to account for the pH-dependence of ATPase activity and for the effects of Mg^{2+} on activity at high pH. It is shown that effects of Mg^{2+} on measurements of ATPase activity as a function of ATP concentration at pH 8.0 and pH 8.5 are consistent with binding of Mg^{2+} to the Ca^{2+}-binding sites on the phosphorylated ATPase, such binding inhibiting dephosphorylation of the ATPase. It is also shown that slow dissociation of Ca^{2+} from the phosphorylated ATPase is consistent with the previously published model.

INTRODUCTION

In previous papers we have developed a kinetic model for the $Ca^{2+} + Mg^{2+}$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) purified from rabbit muscle sarcoplasmic reticulum (SR) (Froud & Lee, 1986*a*,*b*; Gould *et al.*, 1986; Stefanova *et al.*, 1987). The model is based on that proposed by de Meis & Vianna (1979), involving two main conformational states for the ATPase, E1 and E2 (see Scheme 1 below). In the E1 conformation, the two Ca^{2+}-binding sites per ATPase molecule are of high affinity and are exposed to the outer (cytoplasmic) side of the SR membrane, whereas in the E2 conformation the two sites are of low affinity and are exposed to the inside of the SR. Further, in the E1 conformation the ATPase can be phosphorylated by MgATP, whereas in the E2 conformation it can be phosphorylated by phosphate (de Meis, 1981).

One prominent feature of the steady-state kinetics of the ATPase is the marked pH-dependence of ATPase activity, a plot of ATPase activity against pH being bell-shaped with a maximum at about pH 7.5 (de Meis, 1981). The observed decrease in ATPase activity at acid pH can be interpreted quantitatively (Gould *et al.*, 1986) in terms of the known decrease in the rate of dephosphorylation of the phosphorylated ATPase with decreasing pH (de Meis, 1981; Inesi *et al.*, 1984; Froud & Lee, 1986*b*). There is, however, less certainty as to the step or steps responsible for the decrease in ATPase activity at pH values greater than pH 7.5.

For a pH-sensitive step to have a marked effect on steady-state ATPase activity it is generally necessary for that step to be slow and at least partly rate-controlling. Under conditions where the concentrations of Ca^{2+} and MgATP are not limiting, we have shown that there are two main rate-controlling steps in the reaction sequence, dephosphorylation of the phosphorylated ATPase (E2P) and the rate of the change $Ca_2E1'P \rightarrow E2P$ (see Scheme 2 below; Gould *et al.*, 1986; Stefanova *et al.*, 1987). For the $Ca_2E1'P \rightarrow E2P$ change, we suggested that the slow step was the conformational transition $Ca_2E1'P \rightarrow Ca_2E2'P$ (k_1) rather than dissociation of Ca^{2+} from $Ca_2E2'P$ (k_2 to k_4; Gould *et al.*, 1986). We therefore suggested that the $Ca_2E1'P \rightarrow Ca_2E2'P$ transition (k_1) was the required second pH-sensitive step, the rate of this step decreasing with increasing pH (Gould *et al.*, 1986).

The effect of pH on the rate of the $Ca_2E1'P \rightarrow E2P$ change has subsequently been measured directly by a rapid filtration method in which the time course of release of Ca^{2+} from the phosphorylated ATPase is observed. Wakabayashi *et al.* (1986) and Champeil & Guillain (1986) showed that the rate of release of Ca^{2+} was only slightly dependent on pH, the rate in fact increasing slightly from pH 6 to pH 8. Further, Bishop & Al-Shaw (1988) showed that the inhibition of ATPase activity observed at high pH was very dependent on the concentration of Mg^{2+} present in the system, with ATPase activities measured at pH 8 in the presence of low concentrations of Mg^{2+} being higher than those measured at pH 7, whereas at concentrations of Mg^{2+} above 10 mM activities measured at pH 8 were very much less than those measured at pH 7. Bishop & Al-Shaw (1988) suggested that inhibition of ATPase activity by Mg^{2+} at pH 8 could follow from binding of Mg^{2+} to the Ca^{2+}-binding sites on E2P. A similar suggestion has been made by Wakabayashi *et al.* (1987).

A second necessary modification of our previous model (Gould *et al.*, 1986) follows from recent experiments that suggest that the slow step in the $Ca_2E1'P \rightarrow E2P$ change is probably dissociation of Ca^{2+} from the phosphorylated ATPase (Khananshvili & Jencks, 1988). A variety of experiments have suggested that binding of Ca^{2+} to the E1 conformation of the ATPase is sequential:

$$E1 \rightarrow CaE1 \rightarrow CaE1' \rightarrow Ca_2E1'$$

with the $CaE1 \rightarrow CaE1'$ transition being relatively slow (Dupont, 1982; Fernandez-Belda *et al.*, 1984; Froud & Lee, 1986*a*). We suggested (Gould *et al.*, 1986) that dissociation of Ca^{2+} from the phosphorylated ATPase occurred through an analogous sequence of reactions as shown in Scheme 2 below.

Khananshvili & Jencks (1988) studied the transport of $^{45}Ca^{2+}$ into either empty SR vesicles or SR vesicles loaded with unlabelled Ca^{2+}, and showed that release of the first Ca^{2+} into the vesicles was relatively fast, but that release of the second Ca^{2+} was slow and exhibited a lag phase. Further, they demonstrated that the release of the second Ca^{2+} was inhibited by high concentrations of Ca^{2+} within the vesicles (Khananshvili & Jencks, 1988). As described by Khananshvili & Jencks (1988), these observations are consistent with the model for Ca^{2+} release that we proposed (Gould *et al.*, 1986). The rate of release of Ca^{2+}

Abbreviations used: ATPase, $Ca^{2+} + Mg^{2+}$-activated ATPase; SR, sarcoplasmic reticulum.

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is slow enough to be partly rate-controlling for ATPase activity assayed under normal conditions (Khananshvili & Jencks, 1988). Further evidence that dissociation of Ca^{2+} is slow comes from experiments by Fernandez-Belda & Inesi (1986) on the formation of ATP on addition of ADP and high concentrations of Ca^{2+} to the ATPase phosphorylated by phosphate. They observed that formation of ATP under these conditions was a relatively slow process (Fernandez-Belda & Inesi, 1986), whereas in terms of our previously published model (Gould *et al.*, 1986) it would be fast, since it was assumed that binding of Ca^{2+} to E2P (k_4) was fast, as was the $Ca_2E2'P \rightarrow Ca_2E1'P$ step (k_1).

In the present paper we study the effect of Mg^{2+} on ATPase activity at high pH in more detail, and show that our previously published model (Gould *et al.*, 1986) can be modified to accommodate the newly published data, with dissociation of Ca^{2+} from the phosphorylated ATPase being slow.

MATERIALS AND METHODS

AnalaR reagents were obtained from BDH Chemicals, and Hepes (Ultrol) was from Calbiochem. $Ca^{2+}+Mg^{2+}$-activated ATPase was prepared from female rabbit (New Zealand White) hindleg muscle as described in Froud & Lee (1986*a*). ATPase activity was determined at 25 °C by using a coupled enzyme assay in a medium, unless otherwise specified, containing 40 mM-Hepes/KOH buffer, 1.02 mM-EGTA, 5 mM-$MgSO_4$, 0.42 mM-phosphoenolpyruvate, 0.15 mM-NADH, pyruvate kinase (30 units) and lactate dehydrogenase (90 units) in a total volume of 2.5 ml. The reaction was started by addition of a portion of 25 mM-$CaCl_2$ to a cuvette containing the ATPase and the other reagents; the concentration of Ca^{2+} was chosen to give maximal ATPase activity at the given pH. ADP production was detected by measurement of NADH oxidation at 340 nm. It was confirmed that the activity of the coupled-enzyme system was in large excess over that of the ATPase under all conditions by demonstrating that addition of ADP resulted in a rapid (< 5 s) decrease in absorbance at 340 nm. Further, increasing the concentrations of the coupling enzymes had no effect on the measured ATPase rates.

Steady-state measurements of enzyme phosphorylation by ATP were carried out in a medium of the following composition: 20 mM-Hepes/KOH buffer, pH 7.2, 5 mM-$MgSO_4$, 100 mM-KCl, 1 mM-$CaCl_2$ and 0.1 mg of ATPase/ml, in a total volume of 1 ml. The reaction was started by addition of 100 μM-[γ-^{32}P]ATP and, after incubation at 25 °C for 10 s, was quenched with ice-cold 25% (w/v) trichloroacetic acid and 0.2 M-potassium phosphate. The quenched protein was allowed to form a precipitate by incubating the mixture on ice for 15 min. The precipitate was collected by filtration through Whatman GF/C glass-fibre filters, washed three times with 15 ml of cold 12% trichloroacetic acid/0.2 M-potassium phosphate, and finally its radioactivity was counted in 4 ml of Labscint. Protein determinations showed that the recovery of protein in the filtration step was greater than 90%.

Kinetic simulations were carried out by using the FACSIMILE program (Chance *et al.*, 1977) running on an IBM 3090 computer.

RESULTS

ATPase activities measured at high (2.1 mM) concentrations of ATP are highly variable among different preparations of the ATPase (Fig. 1; Gould *et al.*, 1986). To determine the concentrations of active ATPase in the preparations we measured extents of phosphorylation of the ATPase under conditions where simulations suggest that the ATPase should be > 97% phosphorylated (100 μM-[γ-^{32}P]ATP, pH 7.2, in the presence of 1 mM-$CaCl_2$). As shown in Fig. 1, extents of phosphorylated ATPase measured under these conditions are also highly variable among different preparations, with values between 0.25 and 0.6 of that expected for phosphorylation of a 100% pure and active preparation of the ATPase (8.7 nmol/mg of protein). SDS/PAGE of all preparations show the ATPase to be the major species (see Gould *et al.*, 1987*b*), so that the relatively low extents of phosphorylation observed in these experiments presumably reflect damage to the ATPase during preparation. There is a reasonable correlation between maximal extent of phosphorylation and steady-state ATPase activities measured at high concentrations of ATP, although significant differences in ATPase activity are observed among different preparations showing the same maximal extents of phosphorylation (Fig. 1).

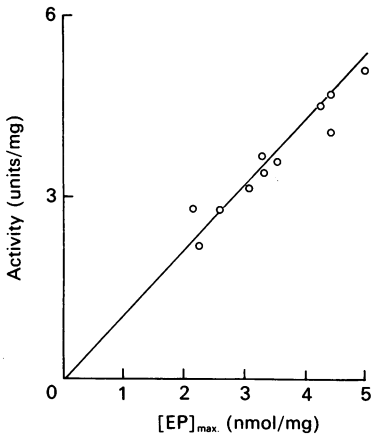


Fig. 1. Relationship between maximal extent of phosphorylation and ATPase activity

For 11 different preparations of the ATPase, maximal extents of phosphorylation (nmol of EP/mg of protein) were determined under the conditions described in the Materials and methods section and plotted against ATPase activity (units/mg of protein) measured at 25 °C and pH 7.2 at an ATP concentration of 2.1 mM, other conditions being as described in the Materials and methods section.

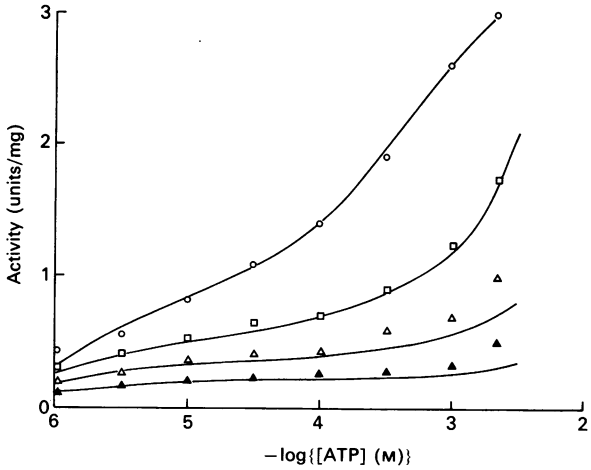


Fig. 2. Effect of Mg^{2+} on ATPase activity at pH 8

The experimental data show the ATPase activity as a function of −log[ATP] at 25 °C and pH 7.2, $[Mg^{2+}]$ = 5 mM and $[K^+]$ = 12 mM (○), or pH 8.0, $[K^+]$ = 20 mM and $[Mg^{2+}]$ = 5 mM (□), 10 mM (△) or 20 mM (▲). The lines are simulations with the parameters in Table 1, an ATPase purity of 0.45 and an ATP stimulation factor of 7.

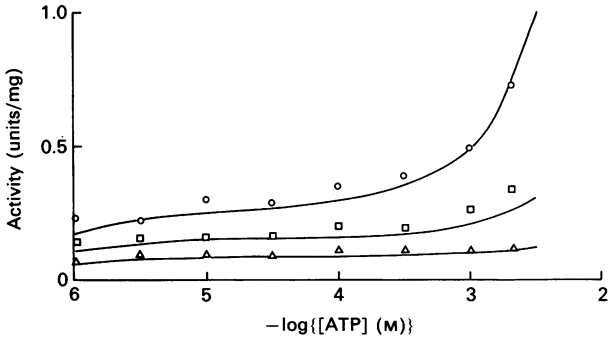


Fig. 3. Effect of Mg^{2+} on ATPase activity at pH 8.5

The experimental data show the ATPase activity as a function of $-\log[ATP]$ at 25 °C and pH 8.5, $[K^+] = 20$ mM and $[Mg^{2+}] = 5$ mM (○), 10 mM (□) or 20 mM (△). The lines are simulations with the parameters in Table 1, an ATPase purity of 0.5 and an ATP stimulation factor of 8.

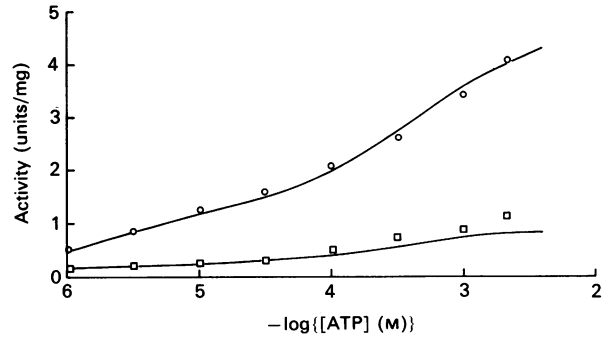


Fig. 4. ATPase activity at pH 6.0

The experimental data show the ATPase activity as a function of $-\log[ATP]$ at 25 °C, $[Mg^{2+}] = 5$ mM, $[K^+] = 12$ mM and pH 7.2 (○) or pH 6.0 (□). The lines are simulations with the parameters in Table 1, an ATPase purity of 0.62 and an ATP stimulation factor of 7.

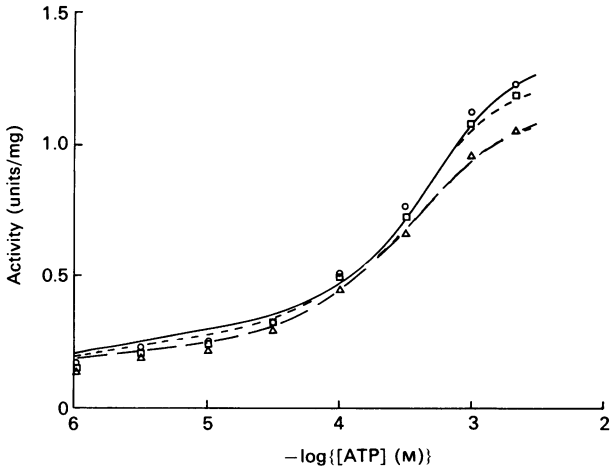


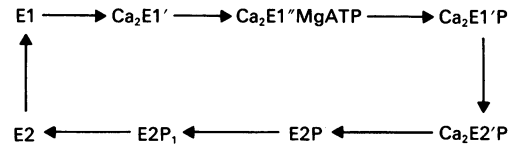
Fig. 5. Effect of K^+ on ATPase activity at pH 6.0

The experimental data show the ATPase activity as a function of $-\log[ATP]$ at 25 °C, $[Mg^{2+}] = 5$ mM, pH 6.0 and $[K^+] = 20$ mM (△), 50 mM (□) or 100 mM (○). The lines are simulations with the parameters in Table 1, an ATPase purity of 0.62 and an ATP stimulation factor of 11 and: ——, $[K^+] = 20$ mM; – – – –, $[K^+] = 50$ mM; ——, $[K^+] = 100$ mM.

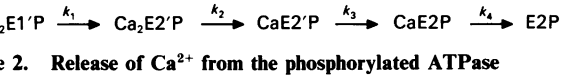
ATPase activities were determined as a function of ATP concentration at maximally stimulating concentrations of Ca^{2+}. The dependence of ATPase activity on the concentration of ATP is complex, with ATP at high concentrations producing a stimulation of activity (Fig. 2). As shown in Fig. 2, activities measured at ATP concentrations between 1 and 2100 μM are less at pH 8 than at pH 7.2, for Mg^{2+} concentrations between 5 and 20 mM, activities decreasing with increasing concentration of Mg^{2+}. Inhibition of ATPase activity is more marked at high concentrations of ATP than at low ones (Fig. 2). A similar effect of Mg^{2+} is observed at pH 8.5, with activities being less than at pH 8.0 (Fig. 3). As shown in Fig. 4, ATPase activities measured at pH 6.0 are also less than those measured at pH 7.2, again with more marked inhibition at high concentrations of ATP than at low ones. In agreement with the results obtained by Bishop & Al-Shaw (1988), increasing the Mg^{2+} concentration from 5 to 20 mM had little effect on ATPase activity at pH 6 (results not shown, but see Fig. 9). It was suggested previously that K^+ and H^+ had inter-related effects on ATPase activity (Gould *et al.*, 1986). Fig. 5 shows ATPase activities measured at pH 6 as a function of K^+ concentration.

DISCUSSION

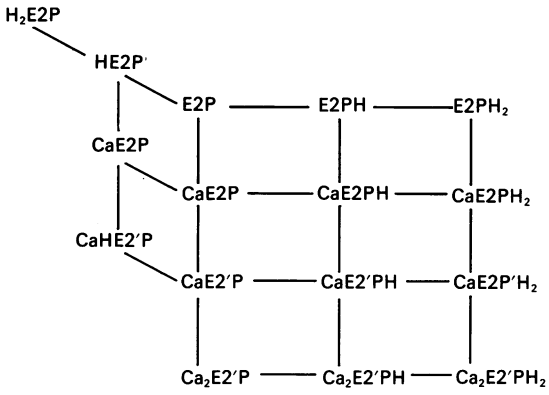
The majority of experiments on the kinetics of the ATPase have been interpreted in terms of the E1/E2 model shown in simplified form in Scheme 1. The Scheme proposes two major conformational states E1 and E2 for the ATPase, differing in the affinity and orientation of the Ca^{2+}-binding sites. Following binding of Ca^{2+} and MgATP to the E1 conformation, the ATPase is phosphorylated to give $Ca_2E1'P$ in which two Ca^{2+} ions per ATPase molecule are occluded and unable to dissociate to the outside of the SR (Dupont, 1980). The ATPase can then undergo a conformational change to a form ($Ca_2E2'P$) with Ca^{2+}-binding sites of low affinity from which Ca^{2+} can dissociate to the inside of the SR (Scheme 2). Following dephosphorylation to give the E2 conformation, the ATPase can recycle to E1. Stahl & Jencks (1987), however, have argued against the E1/E2 model, and have suggested that dephosphorylation of the phosphorylated ATPase (E2P in the nomenclature of Scheme 1) leads directly to a species whose Ca^{2+}-transport sites face the cytoplasm (E1 in the nomenclature of Scheme 1) without any need for an intermediate E2 form. In our simulations of the kinetics of the ATPase we have assumed the E2 → E1 step to be fast and thus without any direct effect on the steady-state kinetics of the ATPase (Gould *et al.*, 1966; Stefanova *et al.*, 1987). However, it was necessary to propose a pH-dependent equilibrium between E2 and E1 forms of the ATPase to account for effects of pH on the fluorescence of the ATPase labelled with fluorescein isothiocyanate (Pick, 1982; Froud & Lee, 1986a; Lee, 1988) and to interpret effects of pH on Ca^{2+} binding to the ATPase (Froud



Scheme 1. Simplified reaction scheme for the ATPase, as proposed by de Meis & Vianna (1979)

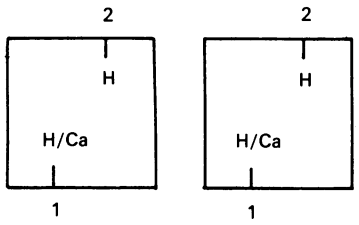


Scheme 2. Release of Ca^{2+} from the phosphorylated ATPase



Scheme 3. Binding of Ca^{2+} to E2P

Binding of Ca^{2+} is competitive with protonation of residue 1 with proton association constant K_{H2}. Binding of Ca^{2+} is non-competitive with protonation of residue 2. For clarity, binding of protons to residue 1 is only indicated for the unprotonated state of residue 2.



Scheme 4. The two Ca^{2+}-binding sites on the ATPase

At each site, two protonatable residues 1 and 2 are proposed. Protonation of site 1 is competitive with binding of Ca^{2+} at that site. Binding of Ca^{2+} is not competitive with protonation of residue 2, although the binding constant for protons at residue 2 depends on the occupancy of the site by Ca^{2+} and, correspondingly, the binding constant for Ca^{2+} depends on the protonation state of residue 2.

& Lee, 1986*a*) and on phosphorylation of the ATPase by phosphate (Froud & Lee, 1986*b*). Further, we have shown that it is possible, by the addition of nonylphenol, to slow the E2 → E1 step sufficiently for it to become a rate-controlling step in the reaction sequence (F. Michelangeli, S. Orlowski, P. Champeil, J. M. East & A. G. Lee, unpublished work).

Stahl & Jencks (1987) have presented experiments that show the presence of a conformational change between binding of MgATP to Ca_2E1' and phosphorylation, possibly corresponding to the relocation of the γ-phosphate of MgATP close to the residue on the ATPase to be phosphorylated; this conformational change has now been incorporated into the model, but is not discussed further in the present paper, since it has no effect on the simulations to be described here.

The main concern of the present paper is with the dissociation of Ca^{2+} from $Ca_2E2'P$ and the possible binding of Mg^{2+} to E2P. In Froud & Lee (1986*a*) and Gould *et al.* (1986) we showed that binding of Ca^{2+} to E1 or to E2P could be described in terms of Scheme 3. In this Scheme we envisage two identical independent Ca^{2+}-binding sites, each containing two ionizable residues, 1 and 2 (Scheme 4). Binding of Ca^{2+} is competitive with protonation of residue 1 (with protonation constant K_{H2}). Binding of Ca^{2+} is, however, not competitive with protonation of residue 2, although the binding constants for protons at this site do depend on the occupation of the site by Ca^{2+}. The possible states of protonation are shown in Scheme 3. We showed that, if it was assumed that protonation constants for residue 2 were the same for all conformational states of the ATPase whereas the protonation constant for residue 1 was different in the E1 and E2 conformations, then it was possible to account both for the pH-dependence of the E1/E2 equilibrium deduced from fluorescence measurements on the ATPase labelled with fluorescein isothiocyanate and for the effect of pH on the binding of Ca^{2+} to the ATPase (Froud & Lee, 1986*a*; Lee, 1988). The model is equally consistent with sequential binding to two Ca^{2+}-binding sites, on removal of the statistical factors of 2 (Roberts, 1977) introduced for the case of two identical Ca^{2+}-binding sites. The derived protonation constants for the E1 and E2 (or E2P) forms of the ATPase are given in Froud & Lee (1986*a*) and Gould *et al.* (1986).

Champeil & Guillain (1986) have measured directly the rate of release of Ca^{2+} bound to the ATPase on addition of MgATP, corresponding to the $Ca_2E1'P \rightarrow E2P$ change (Scheme 2), and shown the release to be sufficiently slow to be rate-controlling for ATP hydrolysis by the ATPase. In Gould *et al.* (1986) we suggested that the slow step in the $Ca_2E1'P \rightarrow E2P$ sequence was the $Ca_2E1'P \rightarrow Ca_2E2'P$ step (k_1), with dissociation of Ca^{2+} from $Ca_2E2'P$ being fast. If this were so, then, in a single-turnover experiment, both Ca^{2+} ions bound to the ATPase would be released into the interior of SR vesicles at the same rate [determined by the rate of the $Ca_2E1'P \rightarrow Ca_2E2'P$ step (k_1)]. Experiments by Khananshvili & Jencks (1988; Figs. 6 and 7) show that this is not so; release of the second Ca^{2+} ion is slower than that of the first and exhibits a marked delay (Fig. 6). This pattern of Ca^{2+} release from $Ca_2E2'P$ markedly resembles the pattern of Ca^{2+} release from Ca_2E1' (Dupont, 1982; Fernandez-Belda *et al.*, 1984), where release of the second Ca^{2+} ion also exhibits a delay, attributed to a relatively slow conformation change $CaE1' \rightarrow CaE1$ (Froud & Lee, 1986*a*). The rate of this step must be stimulated by binding of MgATP, since the rate of phosphorylation of the ATPase following addition of Ca^{2+} to E1MgATP is faster than the rate of the $CaE1 \rightarrow CaE1'$ step (Stahl & Jencks, 1984; Pickart & Jencks, 1984). Similarly, Champeil & Guillain (1986) have shown that the rate of the

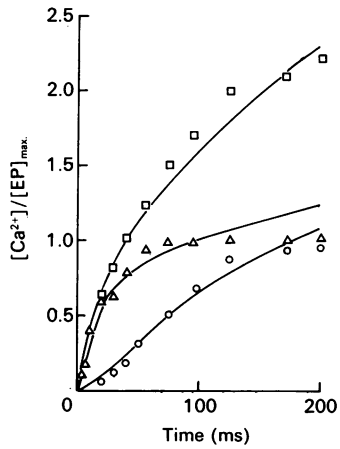


Fig. 6. Dissociation of Ca^{2+} from phosphorylated ATPase

The experimental data from Khananshvili & Jencks (1988) show the accumulation of Ca^{2+} into empty SR vesicles on addition of 0.25 mM-ATP to SR incubated with 28 μM-Ca^{2+} and 0.9 mM-Mg^{2+}, at 100 mM-KCl, pH 7.0 (□). ○ and △ symbols are derived data showing the accumulation of the first and the second Ca^{2+} ions, corrected for continued turnover of the ATPase. The curves are simulations of the accumulation of total Ca^{2+} and of the first and second Ca^{2+} ions with the parameters in Table 1, and a first-order rate constant for leak of Ca^{2+} from the vesicles of 1.2×10^{-3}, with an internal volume for the SR vesicles of 2 μl/mg of protein.

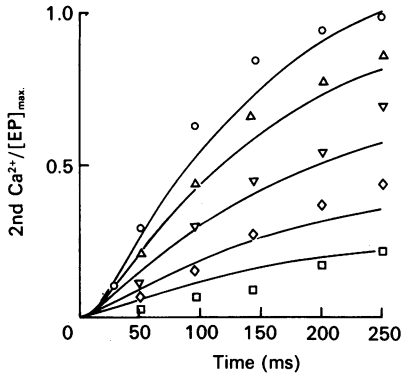


Fig. 7. Effect of internal Ca^{2+} concentration on rate of dissociation of Ca^{2+} from phosphorylated ATPase

The experimental data from Khananshvili & Jencks (1988) show the internalization of the second Ca^{2+} ion into SR vesicles loaded with the given concentrations of Ca^{2+}, on addition of 0.25 mM-ATP to SR incubated with 28 μM-Ca^{2+} and 0.9 mM-Mg^{2+}, at 100 mM-KCl, pH 7.0. Internal Ca^{2+} concentrations: ○, 0.03 mM; △, 1.0 mM; ▽, 3.0 mM; ◇, 7.0 mM; □, 13.0 mM. The curves are simulations with the parameters in Table 1, and a first-order rate constant for leak of Ca^{2+} from the vesicles of 1.2×10^{-3}, with an internal volume for the SR vesicles of 2 μl/mg of protein.

Table 1. Kinetic parameters for ATPase at 25 °C

Parameters were obtained by simulation as described in the text. Values for parameters not listed were taken from Gould *et al.* (1986) and Stefanova *et al.* (1987).

Reaction	Equilibrium constant	Backward rate constant (s^{-1})
$E2P + Ca^{2+} \rightleftharpoons CaE2P$	345	2.9×10^5
$E2PH + Ca^{2+} \rightleftharpoons CaE2PH$	345	2.9×10^5
$E2PH_2 + Ca^{2+} \rightleftharpoons CaE2PH_2$	345	2.9×10^5
$CaE2P \rightleftharpoons CaE2'P$*†	1.1	15.3
$CaE2PH \rightleftharpoons CaE2'PH$*†	3.0	15.3
$CaE2PH_2 \rightleftharpoons CaE2'PH_2$*†	0.7	15.3
$CaE2'P + Ca^{2+} \rightleftharpoons Ca_2E2'P$‡	2.1×10^3	715
$CaE2'PH + Ca^{2+} \rightleftharpoons Ca_2E2'PH$‡	87	715
$CaE2'PH_2 + Ca^{2+} \rightleftharpoons Ca_2E2'PH_2$‡	45	715
$E2P + Mg^{2+} \rightleftharpoons E2PMg$	2×10^4	Fast

* Rates for unprotonated aspartyl phosphate; rates for K^+-bound form [E2(PK)] 25% of those listed and zero rates for protonated form [E2(PH)].

† Rates for MgATP-bound forms vary between preparations, being typically 6–11 times the listed rates (actual stimulation factors given in Figure legends).

‡ Rates for MgATP-bound forms 5 times the values listed.

$Ca_2E1'P \rightarrow E2P$ change is increased by high concentrations of MgATP, and Khananshvili & Jencks (1988) have shown that this is due to an increased rate of release of Ca^{2+} from the phosphorylated ATPase.

The experimental data of Khananshvili & Jencks (1988) can be used, in conjunction with our previously derived protonation constants (Gould *et al.*, 1986), to fix Ca^{2+}-binding constants and rates in terms of Schemes 2 and 3. The experiments of Khananshvili & Jencks (1988) involve measuring the rate of release of $^{45}Ca^{2+}$ into the interior of SR vesicles following the addition of MgATP to either empty vesicles or vesicles loaded with unlabelled Ca^{2+}. These experiments are, in general, not simple single-turnover experiments, but show continued accumulation of Ca^{2+} due to continued turnover of the ATPase. The extent of the accumulation of Ca^{2+} following the first turnover will depend on the rate of leak of Ca^{2+} from the SR vesicles, which can be relatively fast (see Gould *et al.*, 1987*a*). For the simulations reported here, leak was simulated as a simple first-order process, whose rate was chosen to match the experimentally determined rate of accumulation of Ca^{2+}. As shown in Fig. 6, the experimental data on Ca^{2+} accumulation can be simulated well with the parameters given in Table 1, with the rate of the $Ca_2E1'P \rightarrow Ca_2E2'P$ step (k_1) set fast (1×10^8 s^{-1}) and equal for MgATP-bound and MgATP-free forms; the rate of the back reaction $Ca_2E2'P \rightarrow Ca_2E1'P$ is then equal to 3.3×10^9 s^{-1} from the $Ca_2E1'P/Ca_2E2'P$ equilibrium constant of 0.03 derived in Stefanova *et al.* (1987). Simulations are insensitive to the rate of this step, if it is set fast as done here. Similarly, simulations are insensitive to the rate of binding and release of the first Ca^{2+} ion from $Ca_2E2'P$ (k_4) as long as these are fast; in Table 1, the rate of the forward step has been set at 1×10^8 s^{-1}. The rates of release of the first and second Ca^{2+} ions also match well those derived experimentally, particularly for times less than 100 ms, which correspond largely to single-turnover conditions; the experimental data of Khananshvili & Jencks (1988) for release of the first and second Ca^{2+} ions have been corrected for continued turnover of the ATPase and so diverge at longer times from the simulations that have not been modified in this way. Khananshvili & Jencks (1988) showed that the Ca^{2+}-release data shown in Fig. 6 were consistent with sequential release of two Ca^{2+} ions with effective rate constants of 34 s^{-1} and 17 s^{-1} for the release of the first and second Ca^{2+} ions respectively. These numbers can be compared with the effective rate constants calculated under the

Table 2. Effective rates for binding of Ca^{2+} to the phosphorylated ATPase

Rates for Ca^{2+} binding and dissociation were calculated by using the parameters listed in Table 1, for pH 7.0, [KCl] = 100 mM and [Mg^{2+}] = 5 mM.

Reaction	Effective rate constants: Forward	Back
$E2P + Ca^{2+} \rightleftharpoons CaE2P$	6.7×10^7 $M^{-1} \cdot s^{-1}$	2.9×10^5 s^{-1}
$CaE2P \rightleftharpoons CaE2'P$	4.4 s^{-1}	4.7 s^{-1}
$CaE2'P + Ca^{2+} \rightleftharpoons Ca_2E2'P$	1.7×10^4 $M^{-1} \cdot s^{-1}$	1.4×10^3 s^{-1}
$E2PMgATP + Ca^{2+} \rightleftharpoons CaE2PMgATP$	6.7×10^8 $M^{-1} \cdot s^{-1}$	2.9×10^6 s^{-1}
$CaE2PMgATP \rightleftharpoons CaE2'PMgATP$*	31.1 s^{-1}	33.1 s^{-1}
$CaE2'PMgATP + Ca^{2+} \rightleftharpoons Ca_2E2'PMgATP$	8.5×10^4 $M^{-1} \cdot s^{-1}$	7.1×10^3 s^{-1}

* With an ATP stimulation factor of 7.

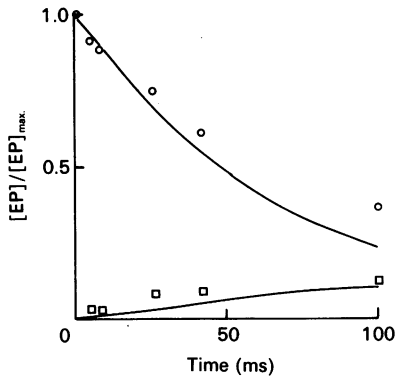


Fig. 8. Phosphoenzyme decay and formation of ATP on addition of ADP and Ca^{2+} to the ATPase phosphorylated with phosphate

The experimental data from Fernandez-Belda & Inesi (1986) show the rate of phosphoenzyme decay (○) and the rate of formation of ATP (□) on addition of 15 mM-Ca^{2+} and 2.0 mM-ADP to the ATPase previously phosphorylated with phosphate, in buffer at pH 6.8, containing 80 mM-KCl and 5 mM-Mg^{2+}. The curves are simulations with the parameters in Table 1.

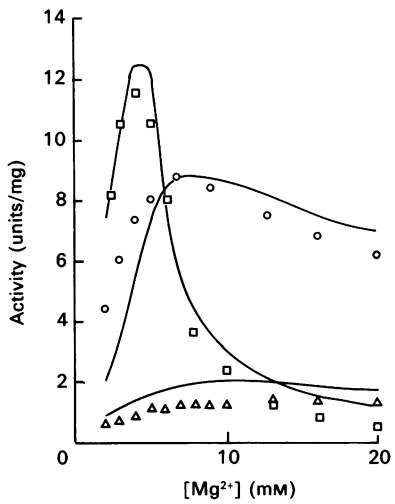


Fig. 9. Effect of Mg^{2+} on ATPase activity

The experimental data from Bishop & Al-Shaw (1988) show ATPase activity as a function of Mg^{2+} concentration in 80 mM-KCl, at 5 mM-ATP with an optimal Ca^{2+} concentration, at pH 6.0 (△), 7.0 (○) or 8.0 (□). The curves showing inhibitory effects at high concentrations of Mg^{2+} are simulations with the parameters in Table 1, at ATPase purity of 0.93 and an ATP stimulation factor of 20. Inhibitory effects seen at low concentrations of Mg^{2+} were calculated as described in the text.

conditions of this experiment, calculated from the parameters given in Table 1 (Table 2). The rate of dissociation of the second Ca^{2+} ion will be dominated by the rate of the CaE2′P → CaE2P transition (k_3), which is calculated to be 4.7 s^{-1} and 33.1 s^{-1} for the MgATP-free and MgATP-bound forms respectively. The rate of dissociation of the first Ca^{2+} ion (k_2; 1.4×10^3 s^{-1} and 7.1×10^3 s^{-1} for the MgATP-free and MgATP-bound forms respectively) is very much greater than the effective rate estimated by Khananshvili & Jencks (1988), but in our model the effective rate of dissociation of the first Ca^{2+} will be decreased as a consequence of the Ca_2E2′P → Ca_2E1′P transition (k_1). Applying the quasi-equilibrium approach (Cha, 1968), with an equilibrium constant Ca_2E2′P/Ca_2E1′P of 0.03 (Stefanova *et al.*, 1987), the effective rate of dissociation of the first Ca^{2+} ion becomes 42 s^{-1}. Differences in the rates of the CaE2′P → CaE2P and CaE2′P + Ca^{2+} → Ca_2E2′P transitions between Tables 1 and 2 reflect the effect of K^+ (see below) and the statistical factors of 2 respectively.

An important feature of the sequential model we proposed for release of Ca^{2+} from Ca_2E2′P was that release of the second Ca^{2+} ion should be inhibited by high concentrations of Ca^{2+} within the SR (Gould *et al.*, 1986). This was indeed observed by Khananshvili & Jencks (1988), and Fig. 7 shows that the Ca^{2+}-concentration-dependence of this effect is well matched by the parameters given in Table 1.

Fernandez-Belda & Inesi (1986) studied the rate of formation of ATP on simultaneous addition of Ca^{2+} and ADP to the ATPase phosphorylated with phosphate in the absence of Ca^{2+}. Under these conditions, formation of ATP was found to be much slower than that following addition of ADP to the enzyme phosphorylated with acetyl phosphate in the presence of Ca^{2+}. Phosphorylation with phosphate in the absence of Ca^{2+} will give E2P, whereas phosphorylation with acetyl phosphate will give Ca_2E1′P (Scheme 1). Since the form of the phosphorylated ATPase that is ADP-reactive is Ca_2E1′P, Ca_2E1′P will be able to react rapidly with added ADP, whereas E2P must first bind Ca^{2+}, a slow step, before it can undergo the Ca_2E2′P → Ca_2E1′P transition and become ADP reactive. Fig. 8 shows that the experimental data on the effect of addition of Ca^{2+} and ADP to E2P fit well to the parameters given in Table 1.

Champeil & Guillain (1986) showed that the rate of release of Ca^{2+} on addition of ATP to the ATPase increased slightly from pH 6.0 to pH 8.0, in agreement with observations made by Wakabayashi *et al.* (1986). Champeil & Guillain (1986) also showed that K^+ significantly slowed the rate of release of Ca^{2+}. In our previous model (Stefanova *et al.*, 1987), these effects were attributed to effects on the rate of the Ca_2E1′P → Ca_2E2′P transition (k_1). On the basis of studies of the effects of H^+ and K^+ on phosphorylation of the ATPase by phosphate, it was suggested that H^+ and K^+ could bind competitively to the phosphorylated aspartic acid residue on the phosphorylated ATPase (Froud & Lee, 1986*b*). Effects of H^+ and K^+ on the rate of the Ca_2E1′P → Ca_2E2′P transition (k_1) were then attributed to this binding (Stefanova *et al.*, 1987). Since the slowest step in the Ca_2E1′P → E2P sequence is now suggested to be the conformation change CaE2′P → CaE2P (k_3; Table 1), it is suggested that binding of H^+ and K^+ to the phosphorylated ATPase affects the rate of this step, rather than that of the Ca_2E1′P → Ca_2E2′P step (Table 1); similar effects of binding of H^+ and K^+ on the rates of the other steps involved in dissociation of Ca^{2+} from Ca_2E2′P are possible, but would have no significant effect on the simulations published here.

An important parameter in determining steady-state ATPase activities is the proportion of the ATPase in the active form. As shown in Fig. 1, this varies considerably among different preparations. In simulations of ATPase activity it is therefore necessary to treat the proportion of active ATPase as a variable, as is done in analyses of phosphorylation studies of the ATPase. As shown in Fig. 1, although there is a reasonable correlation between measured ATPase activities at high (2.1 mM) concentrations of ATP and the maximal extent of phosphorylation of that preparation, the correlation is not perfect, and preparations showing the same maximal extents of phosphorylation can show significantly different activities. This has been attributed to different extents of stimulation of the Ca_2E1′P → E2P change by MgATP (Gould *et al.*, 1986). In our previous model, where the slow step in this sequence was believed to be the Ca_2E1′P → Ca_2E2′P step (k_1), we attributed differences to different extents of stimulation of this step by MgATP (Gould

et al., 1986); we now assume it to be due to differences in the extent of stimulation of the $CaE2'P \rightarrow CaE2P$ step (k_3) by MgATP (referred to in the Figure legends as the 'ATP stimulation factor') (Table 1).

Bishop & Al-Shaw (1988) and Wakabayashi *et al.* (1987) have shown that ATPase activity at pH 8 is strongly dependent on the concentration of Mg^{2+} and have attributed this to binding of Mg^{2+} to the Ca^{2+}-binding sites on E2P. This effect of Mg^{2+} is much more marked at pH 8 than at pH 7.2 (compare Fig. 2 with Fig. 1 in Stefanova *et al.*, 1987; see also Fig. 9), suggesting that binding of Mg^{2+} to E2P is pH-dependent. As described elsewhere (Stefanova *et al.*, 1987; Lee, 1987), binding of Mg^{2+} and MgATP to the phosphorylated ATPase is competitive and, since, as described above, binding of MgATP to the phosphorylated ATPase increases the rate of dissociation of Ca^{2+}, high concentrations of Mg^{2+} will decrease ATPase activity by decreasing the binding of MgATP; this effect largely explains the inhibition of ATPase activity at pH 7.0, but is too small to explain the effects observed at pH 8.0 (Fig. 9). The pattern of inhibition caused by high concentrations of Mg^{2+} at pH 8.0 can, however, be simulated assuming that binding of a single Mg^{2+} ion to E2P with unprotonated Ca^{2+} sites (Scheme 3 and Table 1) prevents dephosphorylation of E2P (Fig. 9); the effect of such binding will be much more marked at pH 8.0 than at pH 7.0.

The pattern of effects seen by Bishop & Al-Shaw (1988) at low concentrations of Mg^{2+} are more complex to interpret. At low concentrations of Mg^{2+}, significant amounts of CaATP will be produced, and CaATP is known to phosphorylate the ATPase with a rate and affinity different to those of MgATP (Shigekawa *et al.*, 1983; Yamada *et al.*, 1986). Further, it has been shown that the affinity of the ATPase for Ca^{2+} is affected by changed ionic conditions, including effects of low concentrations of Mg^{2+} (Scofano *et al.*, 1985), probably due to effects of membrane surface charge sensed at the Ca^{2+}-binding sites (Rooney & Lee, 1983). Taking these effects into account, a reasonable fit to the experimental data can also be obtained at low concentrations of Mg^{2+} (Fig. 9; F. Michelangeli, J. Colyer, J. M. East & A. G. Lee, unpublished work).

As shown in Figs. 2 and 3, the inhibition of ATPase activity by high concentrations of Mg^{2+} at pH 8.0 and 8.5 as a function of the concentration of ATP can be simulated well with the parameters derived here (Table 1). The same parameters describe well the inhibition of ATPase activity at acid pH values (Fig. 4), where high concentrations of Mg^{2+} have relatively little effect (Fig. 9). It is known that increasing concentrations of K^+ at pH 6.0 have very marked effects on the rate of dephosphorylation of the phosphorylated ATPase (Inesi & Hill, 1983; Inesi *et al.*, 1984). Since at pH 6.0 dephosphorylation is a very slow step in the reaction sequence, K^+ might have been expected to have a very marked effect on ATPase activity at pH 6.0; in fact, as shown in Fig. 5, the effect is relatively small. As described above, in the simulations this follows from the inhibitory effect of K^+ on the $CaE2'P \rightarrow CaE2P$ transition.

Although we have now shown that the experimental data on the kinetics of the ATPase are consistent with a slow dissociation of Ca^{2+} from $Ca_2E2'P$ and a fast conformational transition $Ca_2E1'P \rightarrow Ca_2E2'P$, we believe that it is still necessary to postulate two distinct conformational states $Ca_2E1'P$ and $Ca_2E2'P$ for the phosphorylated ATPase as in Scheme 1, and that the available data are not consistent with a model in which loss of the first Ca^{2+} ion from the phosphorylated ATPase occurs simultaneously with a change in orientation of the Ca^{2+}-binding sites (i.e. with a direct transition from $Ca_2E1'P$ to $CaE2'P$ in terms of the nomenclature of Scheme 1). In particular, measurements of the extent of phosphorylation of the ATPase by phosphate in the presence of internal phosphate can only be simulated assuming a transition to a second phosphorylated form ($Ca_2E1'P$) following the formation of the initial phosphorylated form $Ca_2E2'P$ (Froud & Lee, 1986*b*).

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REFERENCES

Bishop, J. E. & Al-Shaw, M. K. (1988) J. Biol. Chem. **263**, 1886–1892
Cha, S. (1968) J. Biol. Chem. **243**, 820–825
Champeil, P. & Guillain, F. (1986) Biochemistry **25**, 7623–7633
Chance, E. M., Curtis, A. R., Jones, I. P. & Kirby, C. R. (1977) FACSIMILE: A Computer Program for Flow and Chemistry Simulation, H.M.S.O., London
de Meis, L. (1981) The Sarcoplasmic Reticulum, pp. 1–163, John Wiley and Sons, New York
de Meis, L. & Vianna, A. (1979) Annu. Rev. Biochem. **48**, 275–292
Dupont, Y. (1980) Eur. J. Biochem. **109**, 231–238
Dupont, Y. (1982) Biochim. Biophys. Acta **688**, 75–87
Fernandez-Belda, F. & Inesi, G. (1986) Biochemistry **25**, 8083–8089
Fernandez-Belda, F., Kurzmack, M. & Inesi, G. (1984) J. Biol. Chem. **259**, 9687–9698
Froud, R. J. & Lee, A. G. (1986*a*) Biochem. J. **237**, 197–206
Froud, R. J. & Lee, A. G. (1986*b*) Biochem. J. **237**, 207–215
Gould, G. W., East, J. M., Froud, R. J., McWhirter, J. M., Stefanova, H. I. & Lee, A. G. (1986) Biochem. J. **237**, 217–227
Gould, G. W., McWhirter, J. M., East, J. M. & Lee, A. G. (1987*a*) Biochem. J. **245**, 739–749
Gould, G. W., Colyer, J., East, J. M. & Lee, A. G. (1987*b*) J. Biol. Chem. **262**, 7676–7679
Inesi, G. & Hill, T. L. (1983) Biophys. J. **44**, 271–280
Inesi, G., Lewis, D. & Murphy, A. J. (1984) J. Biol. Chem. **259**, 996–1003
Khananshvili, D. & Jencks, W. P. (1988) Biochemistry **27**, 2943–2952
Lee, A. G. (1987) J. Biomembr. Bioenerg. **19**, 581–603
Lee, A. G. (1988) in Advances in Membrane Fluidity (Aloia, R. C., Curtain, C. C. & Gordon, L. M., eds.), vol. 2, pp. 111–139, Alan R. Liss, New York
Pick, U. (1982) J. Biol. Chem. **257**, 6111–6119
Pickart, C. M. & Jencks, W. P. (1984) J. Biol. Chem. **259**, 1629–1643
Roberts, D. V. (1977) Enzyme Kinetics, p. 184, Cambridge University Press, Cambridge
Rooney, E. K. & Lee, A. G. (1983) Biochim. Biophys. Acta **732**, 428–440
Scofano, H., Barrabin, H., Inesi, G. & Cohen, J. A. (1985) Biochim. Biophys. Acta **819**, 93–104
Shigekawa, M., Wakabayashi, S. & Nakamura, H. (1983) J. Biol. Chem. **258**, 8698–8707
Stahl, N. & Jencks, W. P. (1984) Biochemistry **23**, 5389–5392
Stahl, N. & Jencks, W. P. (1987) Biochemistry **26**, 7654–7667
Stefanova, H. I., Napier, R. M., East, J. M. & Lee, A. G. (1987) Biochem. J. **245**, 723–730
Wakabayashi, S., Ogurusu, T. & Shigekawa, M. (1986) J. Biol. Chem. **261**, 9762–9769
Wakabayashi, S., Ogurusu, T. & Shigekawa, M. (1987) J. Biol. Chem. **262**, 9121–9129
Yamada, S., Fujii, J. & Katayama, H. (1986) J. Biochem. (Tokyo) **100**, 1329–1342

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