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Biochimica et Biophysica Acta 1276 (1996) 188–194

BBA Biochimica et Biophysica Acta

Manganese as a cosubstrate for the phosphorylation of the sarcoplasmic reticulum Ca-dependent adenosine triphosphatase with orthophosphate

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Received 4 January 1996; revised 21 May 1996; accepted 28 May 1996

Abstract

The phosphorylation of the sarcoplasmic reticulum Ca-ATPase (EC 3.6.1.38) with P_i was characterized using Mn as a Mg analogue. Steady state and transient fluorescence and radioisotopic techniques were used; the affinities of Mn and P_i for the enzyme and the rate constants of the phosphorylation and dephosphorylation reactions were determined, under several conditions. The reactions were carried out at pH 5.5 to minimize the binding of contaminant Ca to the transport sites, thus avoiding the use of Ca chelators. The apparent affinity of Mn binding at low [Mn] is larger in the absence of P_i (35 μM) than in the presence of saturating P_i (70 μM). On the contrary, the apparent affinity of Mn for the formation of the phosphoenzyme increases, from 1.5 mM to 0.15 mM, upon increasing [P_i] in the millimolar range. The apparent affinty of P_i for the formation of the phosphoenzyme also increases, from 2.2 mM to 0.2 mM, upon increasing [Mn] in the millimolar range. The equilibrium of the phosphoenzyme with the noncovalent Mn · P_i · Enzyme complex favors the covalent species. The simulation of a reaction model including the random binding of 2 Mn and 1 P_i per mol of ATPase and a noncovalent complex in equilibrium with the phosphoenzyme, using a set of equilibrium constants deduced from the results, agree with the experimental data.

Keywords: Sarcoplasmic reticulum; ATPase, Ca-; Manganese

1. Introduction

The transport of two Ca ions by the sarcoplasmic reticulum (SR) Ca-ATPase is coupled to the hydrolysis of an ATP molecule (for a review see Ref. [1]). Mg is the usual cosubstrate of ATP but it can be substituted by other divalent cations [2,3]. Mg also activates or inhibits other steps of the catalytic cycle [4,5]. This cycle is reversible and the phosphorylation of the SR Ca-ATPase with orthophosphate (P_i) is the first step towards the synthesis of ATP from P_i and ADP at the expense of a Ca gradient [6]. Phosphorylation by P_i requires Mg [7,8], but other divalent cations can replace Mg as cosubstrates [9]. The binding of Mg and P_i occurs in random order [8,10], to form a noncovalent ternary complex prior to the formation of covalent phosphoenzyme; the equilibrium largely favors the covalent species [8]. A similar mechanism of random binding has been later proposed for ATP and Mg [11]. The binding of Mg decreases the affinity for P_i and the binding of P_i decreases the affinity for Mg [8].

The stoichiometry of the cations bound to the catalytic site remains an open question. Using a Mg analogue, [^{54}Mn]Mn, it has been shown that one cation per enzyme unit is required to form a covalent phosphoenzyme with ATP [12]. NMR studies on the P-type Na/K-ATPase provided evidence for the binding of a nucleotide and two cations within the catalytic site [13]. More recently, a study of the inactivation of the SR Ca-ATPase by fluoride clearly showed the binding of two Mg ions to the phosphorylation site [14]; Mg_2F_4 would form a complex analogue to the $MgAlF_4$ previously described as a phosphate analogue [15]. In the present study we inquire further in these questions, using Mn as a Mg analogue and combining fluorescence and radioactive techniques to study the phos-

Abbreviations: SRV, sarcoplasmic reticulum vesicles; Ca-ATPase, Ca-dependent adenosinetriphosphatase; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Tris, Tris[hydroxymethyl]aminomethane.

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PII S0005-2728(96)00076-X

phorylation of the Ca-ATPase by P_i. We show that Mn binds to the SR Ca-ATPase as a cosubstrate of P_i. The results indicate that two Mn ions are involved in the phosphoenzyme formation.

2. Materials and methods

2.1. Experimental procedures

SR vesicles from rabbit skeletal muscles were prepared as described by Champeil et al. [16]. The procedure yields a light SR fraction with high Ca-dependent ATPase activity and very low basal ATPase activity measured in the presence of EGTA.

The intrinsic fluorescence of the Ca-ATPase was measured under steady-state and transient conditions. The fluorescence changes were correlated with the interactions of the protein with several ligands. The reactions were always carried out at 20°C and pH 5.5. The fluorescence measurements under steady-state conditions were performed in a Perkin-Elmer MPF 44A spectrofluorometer; the protein concentration was 125 μg/ml. The wavelengths were set at 290 nm for excitation and at 330 nm for emission. Other details are given elsewhere [17]. The kinetics of fluorescence changes were measured with a Biologic stopped-flow apparatus; the tryptophan fluorescence was excited at 290 nm and the emission was recorded through a J324 bandpass filter.

The binding of Mn was measured with [^{54}Mn]Mn. SR vesicles (1 mg in 1 ml) were incubated for 30 s at 20°C in 100 mM Mes-Tris at pH 5.5 and different amounts of [^{54}Mn]$MnCl_2$ with or without P_i, as indicated under results. The media were filtered through Millipore filters (HAWP 0.45 μM), and the retained radioactivity was measured in a gamma scintillation counter. Parallel experiments were made without SR membranes and the radioactivity remaining in the filters was subtracted to the results.

[^{54}Mn]$MnCl_2$ was obtained from New England Nuclear, E.I. Dupont de Nemours, Boston MA. All other reagents were analytical grade.

2.2. Mathematical procedures

Fits and simulations were done with a scientific graphing software (SigmaPlot, Jandel Scientific, Corte Madera).

The theoretical interpretation of steady-state fluorescence experiments was performed using the following scheme where K_i values are dissociation constants. K_{app} values are operatively defined as the ligand concentration giving one half of the maximal responses. Mn and P_i bind at random before covalent phosphoenzyme formation (Scheme 1).

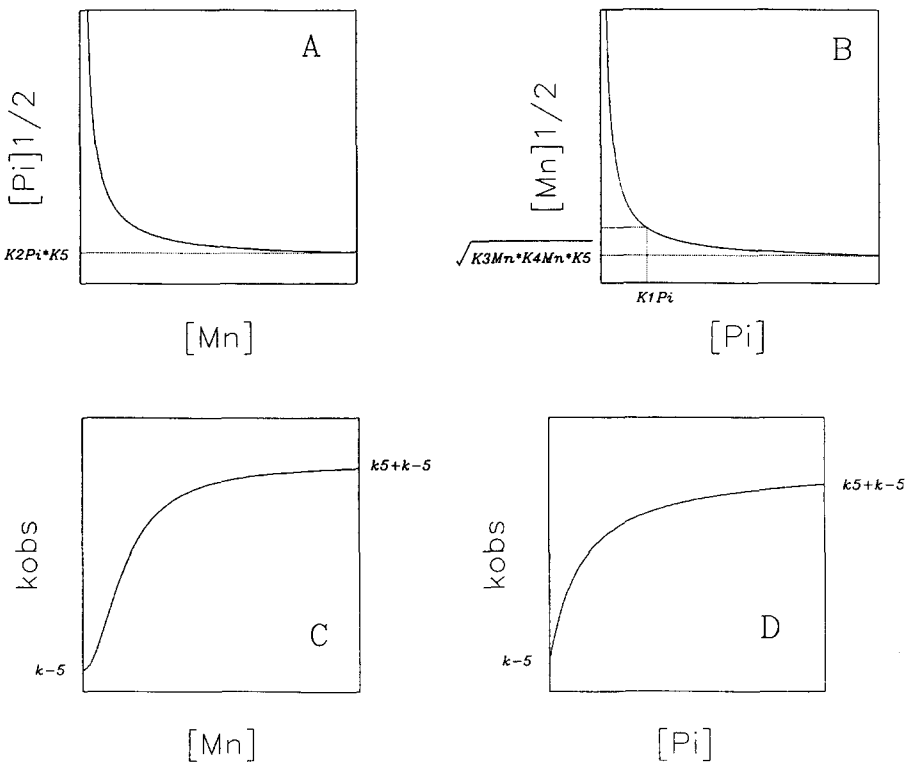
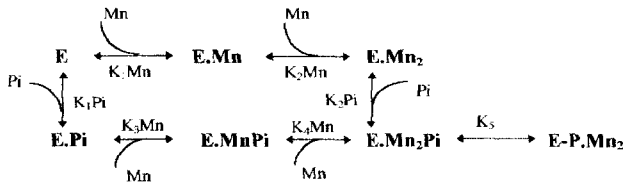


Fig. 1. A and B: simulations of the apparent affinities for P_i and Mn as a function of [Mn] and [P_i], respectively, according to Scheme 1 and Eq. (1) and Eq. (2). C and D: P_i and Mn dependence of the apparent phosphorylation rate constants (k_{obs}) as a function of [Mn] and [P_i], at constant and high [P_i] and [Mn], respectively (Scheme 1 and Eq. (3) and Eq. (4)). The figure shows the pattern of the functions to ilustrate the method of calculation of the K_i values of the model; they apply to any particular numerical value.



Scheme 1.

$K_{app}P_i$ is defined as $[P_i]$ which phosphorylates one half of the enzyme in the presence of different [Mn], as follows:

$$K_{app}P_i = \frac{([E] + [E.Mn] + [E.Mn_2]) \cdot [P_i]}{[E - PMn_2]} = \frac{K_2P_i \cdot K_5 \cdot (K_1Mn \cdot K_2Mn + K_2Mn \cdot [Mn] + [Mn]^2)}{[Mn]^2} \quad (1)$$

$K_{app}Mn$ is defined as [Mn], which acting as a cosubstrate, promotes the phosphorylation of one half of the enzyme in the presence of different $[P_i]$, as follows:

$$K^2_{app}Mn = \frac{[(E) + (E.P_i)] \cdot [Mn]^2}{[E - PMn_2]} = \frac{K_3Mn \cdot K_4Mn \cdot K_5 \cdot (K_1P_i + [P_i])}{[P_i]} \quad (2)$$

The right parts of Eq. (1) and Eq. (2) are obtained by simple algebraic manipulations, from the defined functions and the law of mass action applied to the partial reactions. Fig. 1A and 1B represent the [Mn] and $[P_i]$ dependence of $[P_i]_{1/2}$ $(= K_{app}P_i)$ and $[Mn]_{1/2}$ $(= \sqrt{K^2_{app}Mn})$, respectively, according to Scheme 1 and the above equations. From these curves, it is possible to obtain a value for K_1P_i and combined values for $(K_2P_i{}^* K_5)$ and $(K_3Mn^* K_4Mn^* K_5)$.

To obtain the complete set of values (K_1P_i, K_2P_i, K_1Mn, K_2Mn, K_3Mn, K_4Mn) kinetic data are required ($K_5 = k_{-5}/k_5$). Kinetic fluorescence results were analysed as previously described [8], assuming Scheme 1; they can be summarized as follows:

For constant P_i concentration (Eq. (3))

$$k_{obs} = k_5 \cdot \frac{[Mn]^2}{K^2_{app}Mn + [Mn]^2} + k_{-5}$$

with

$$K^2_{app}Mn = \frac{([E] + [E.P_i]).[Mn]^2}{[E.Mn_2] + [E.Mn_2P_i]} = \frac{K_2P_i \cdot K_1Mn \cdot K_2Mn \cdot (K_1P_i + [P_i])}{K[P_i \cdot (K_2P_i + [P_i])} \quad (3)$$

For constant Mn concentration (Eq. (4))

$$k_{obs} = k_5 \cdot \frac{[P_i]}{K_{app}P_i + [P_i]} + k_{-5}$$

with

$$K_{app}P_i = \frac{([E] + [E.Mn_2]) \cdot [P_i]}{[E.P_i] + [E.Mn_2P_i]} = \frac{K_1P_i \cdot K_2P_i \cdot (K_1Mn \cdot K_2Mn + [Mn]^2)}{K_1Mn \cdot K_2Mn \cdot K_2P_i + K_1P_i \cdot [Mn]^2} \quad (4)$$

Fig. 1C and 1D represent the theoretical ligand dependence of k_{obs} which yields k_{-5}, k_5, and K_5.

The definitions of K_{app} for P_i and Mn (Eq. (1) and Eq. (2)) and the derivations of Eq. (3) and Eq. (4) imply simplifications in the analysis of the model. They have been previously used in analysis of similar reaction models [8], on the basis of the rapid equilibrium of the noncovalent species compared with the slower phosphorylation (k_5) and dephosphorylation (k_{-5}) steps.

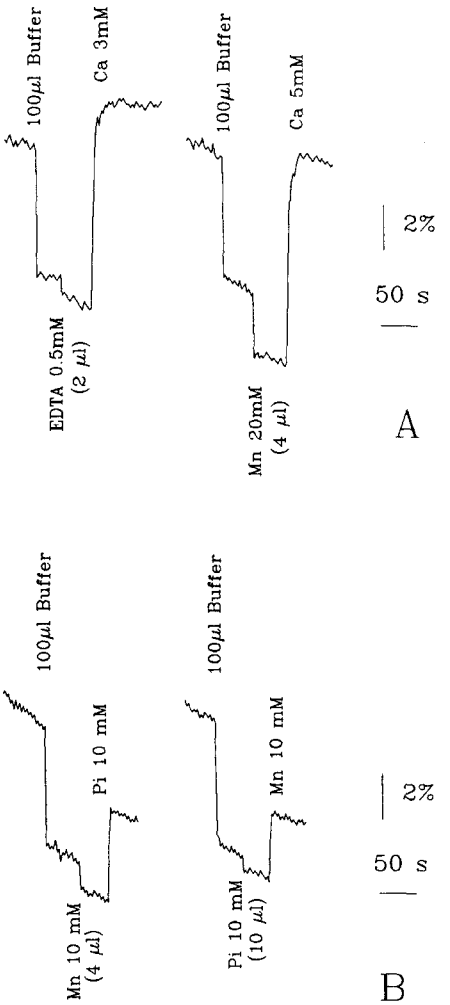


Fig. 2. Intrinsic fluorescence changes induced by addition of several ligands to SR vesicles at acidic pH. SR vesicles were suspended at 0.125 mg protein/ml in a 2 ml medium containing 100 mM MES-Tris buffered at pH 5.5 (20°C). Fluorescence conditions are described under Section 2. A: intrinsic fluorescence changes induced by Ca and Mn. B: intrinsic fluorescence changes induced by Mn and P_i.

3. Results

The effect of Mn and other ligands on the intrinsic fluorescence of the Ca-ATPase was tested at pH 5.5 (Fig. 2). In Fig. 2A, left trace, the addition of EDTA induces a very small decrease of the signal, whereas the subsequent addition of an excess of Ca increases the fluorescence level. The right trace shows that addition of a high Mn concentration induces a fluorescence decrease, and the subsequent addition of Ca increases the fluorescence to a level slightly lower than the one observed in the absence of Mn. In Fig. 2B, the left trace shows that P_i addition after Mn induces a significant enhancement of the fluorescence signal, and the right trace shows that phosphate addition prior to Mn does not change the fluorescence level except for dilution, whereas the later Mn addition now increases the fluorescence level. Fig. 2A demonstrates that at pH 5.5 the ATPase is Ca-free, and Fig. 2B shows a Mn- and P_i-dependent fluorescence enhancement, as previously observed for Mg and P_i [8]. In this later case, their characteristics are coincident with those of the covalent phophoenzyme [8,16]. We conclude that pH 5.5 is a good condition to study the phosphorylation of the Ca-ATPase using Mn as cosubstrate, avoiding the effect of Ca binding to the transport sites without the use of a chelating agent. It should also be taken into account that optimal pH for the phosphorylation of the Ca-ATPase by P_i is around 6.0 [18], and therefore most reports on this reaction have been carried out at acidic pH.

The fluorescence signal observed upon the simultaneous addition of P_i and Mn was titrated as a function of the ligands concentrations (Fig. 3A,B). The data are fitted as sigmoidal functions to determine the apparent affinities for P_i and Mn at various constant Mn and P_i concentrations. Fig. 3C and D shows that those apparent affinities for P_i and Mn increase upon increasing the Mn and P_i concentrations, respectively.

[^{54}Mn]Mn binding was measured in the same medium both in the presence and in the absence of P_i (Fig. 4). The binding curves do not saturate at the highest Mn concentrations, tending to more than 1 mol of bound Mn per mol of ATPase (5–6 nmol/mg) [19]. However, the Scatchard plots support the assumption that the first points indicate the binding of Mn to a first site with affinity ≥ 40 μM in the absence of P_i; the presence of P_i slightly decreases the apparent affinity of the enzyme for the binding of Mn to this site.

The fluorescence experiments give an apparent affinity for Mn higher than mM in the presence of the lowest P_i concentrations (Fig. 3). On the other hand, the radioisotopic experiments yielded a μM affinity for Mn in the absence of P_i (Fig. 4). These different affinities for Mn when P_i is limited or absent can be explained assuming the requirement of two Mn ions to form the covalent phosphoenzyme (Scheme 1). As previously demonstrated [7,10,20], the formation of the covalent phosphoenzyme occurs through a two-step mechanism: the binding of the phosphate and cation(s) leading to a noncovalent intermediate

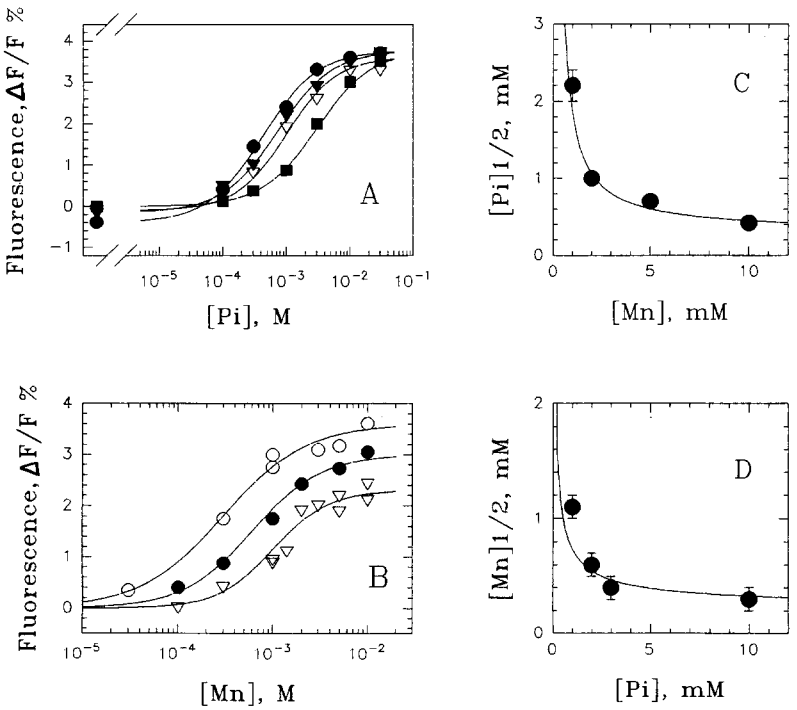


Fig. 3. P_i and Mn dependence of the amplitude of the fluorescence changes (A and B) and of the apparent affinity deduced from those curves using sigmoidal functions (C and D). A: [Mn] = 1 (■), 2 (▽), 5 (▼) or 10 (●) mM. B: [P_i] = 1 (▽), 2 (●) or 10 (○) mM. C and D: solid lines were obtained using the set of constants from Table 1 applied to Eq. (1) and Eq. (2).

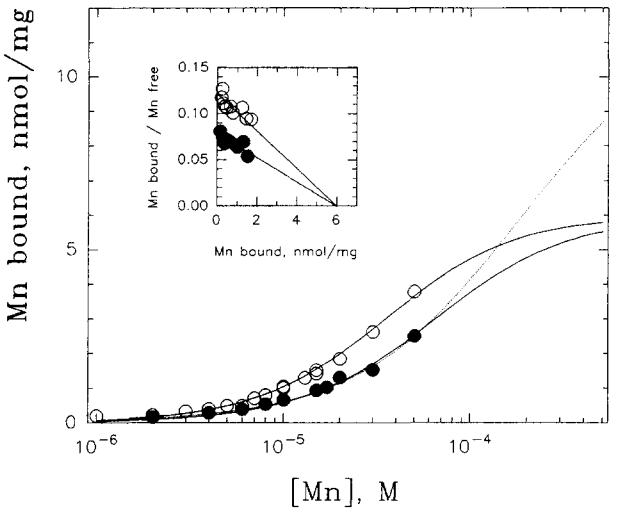


Fig. 4. [Mn] dependence of Mn binding with (●) or without P_i (○). Mn binding was measured as detailed under Section 2. Inset: Scatchard plot of the data.

which is transformed into a covalent complex. The fluorescence levels are an index of the amount of covalent phosphoenzyme, and the radioisotopic measurements indicate the amounts of bound Mn to any species. Assuming the Scheme 1 reaction model, the radioactive data obtained in the presence of high P_i concentration (10 mM) were fitted with a sigmoidal curve drawn up to approx. 2 mol of Mn per mol of ATPase (dotted line in Fig. 4). The apparent affinity for the two Mn in our model, with non-limiting P_i, is the same (0.2 mM) either when obtained from the [^{54}Mn]Mn binding curve or from the fluorescence determinations (Fig. 3B).

Table 1
Equilibrium constants used for the simulation of Scheme 1

$K_1Mn = 0.04–0.10 \cdot 10^{-3}$ M
$K_2Mn = 4–6 \cdot 10^{-3}$ M
$K_1P_i = 6–8 \cdot 10^{-3}$ M
$K_3Mn = 0.15–0.30 \cdot 10^{-3}$ M
$K_4Mn = 8–12 \cdot 10^{-3}$ M
$K_2P_i = 12–16 \cdot 10^{-3}$ M
$K_5 = 0.025$

The equilibrium constant for the step between the noncovalent complex and the covalent species, K_5, is the ratio between the two kinetic constants, k_{-5} over k_5, which have been directly measured or estimated, i.e., 1 s^{-1} and 40 s^{-1}.

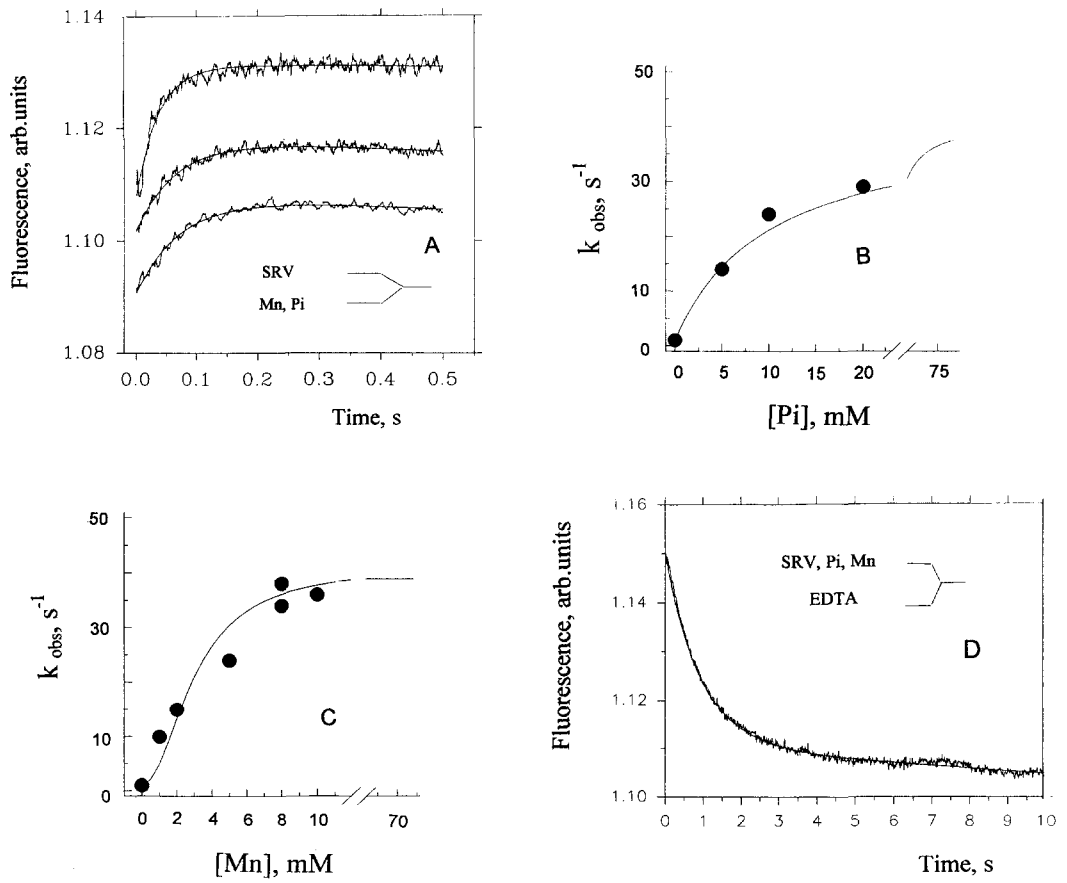


Fig. 5. Kinetics of fluorescence changes induced by Pi and Mn. A: typical stopped-flow records of phosphorylation induced by different [P_i] and [Mn]. B: P_i dependence of the observed rate constant in the presence of 5 mM Mn; C: Mn dependence of the observed rate constant in the presence of 10 mM P_i. D: typical stopped-flow record of dephosphorylation induced by 20 mM EDTA addition to SRV preincubated with 10 mM P_i and 5 mM Mn. Solid lines in B and C were obtained with Eq. (3) and Eq. (4) using the set of constants from Table 1.

The kinetics of the fluorescence changes associated with the phosphorylation and the dephosphorylation of the Ca-ATPase was measured with a stopped flow technique. Fig. 5A depicts some typical records of phosphorylations induced by addition of different P_i and Mn concentrations to the SR suspensions. The rate constant values (k_{obs}) of the fluorescence rise, for the different curves, are obtained assuming single exponential functions. These values are shown for several [P_i] at high [Mn] (Fig. 5B), and for several [Mn] at high [P_i] (Fig. 5C). Fig. 5D shows the time course of dephosphorylation of the Mn.phosphoenzyme; dephosphorylation was started by addition of an excess of EDTA to a phosphoenzyme preformed with Mn as cosubstrate, and the fluorescence decay was recorded.

Eq. (3) and Eq. (4) predict that the phosphorylation rate constant should increase as a function of [P_i] or [Mn] from the true dephosphorylation rate constant to an overestimation of the true phosphorylation rate constant, as schematized in Fig. 1C and D, respectively. The dephosphorylation rate constant obtained from Fig. 5D ($1 s^{-1}$) is consistent with the extrapolated values derived from Fig. 5B and C. The result is also consistent with previous measurements with Mg.phosphoenzymes [8].

Table 1 shows a set of true constants derived from analysis of our data, which fit the Scheme 1 reaction model (Section 2). They are applied to Eqs. (1)–(4), and the functions are shown as continuous traces in Fig. 3C,D and Fig. 5B,C, respectively. The experimental results shown in the same figures fit well with the theoretical functions in all cases.

4. Discussion

The intrinsic fluorescence of the Ca-ATPase has been used to study conformational changes induced by various ligands [21]. It is well known that the intrinsic fluorescence increases due to calcium binding to the transport sites and to phosphorylation with P_i. Since contaminating and endogenous Ca is usually sufficient to saturate the transport sites at neutral pH, a Ca chelating agent is necessary to titrate Ca binding to the transport sites, as well as to observe the conformational change induced by phosphorylation with P_i, since this reaction only takes place in a Ca-deprived enzyme. EGTA has been used with Mg as a cosubstrate of phosphorylation because of its larger affinity for Ca than for Mg, but neither EGTA nor EDTA can be used if we replace Mg by Mn, because of the larger affinity of the chelators for Mn than for Ca. At acidic pH the contaminant Ca is not usually enough to saturate the transport sites [22].

The results from the radioactive and fluorescence determinations are compatible with a reaction model where two Mn ions bind to the catalytic site during phosphorylation with inorganic phosphate (Scheme 1). This stoichiometry is in agreement with previous observations of the simultaneous binding of two Mg and one fluoride to the catalytic site [14]. A second Mn binding to the catalytic site would also correlate with NMR experiments performed on the Na/K-ATPase which show two divalent cation sites at the active site [13].

The first Mn binds with high affinity as revealed by [^{54}Mn]Mn experiments (Fig. 4) and the second Mn binds with lower affinity as revealed by fluorescence experiments at low P_i concentrations (Fig. 3). The high affinity binding of [^{54}Mn]Mn in the absence of P_i (Fig. 4) is not chased by 1 mM Ca (data not shown), thus discarding a binding of Mn to the Ca transport sites, since the apparent affinity of these sites for Ca (at pH 5.5) is approx. 50–60 μM. The very low apparent association constant of Mn and P_i at low pH also discards a significant effect of their interaction on the free Mn and P_i concentrations.

The apparent affinities for P_i and Mn at different [Mn] and [P_i] (Fig. 3C and 3D, respectively), derived from the experimental results (Fig. 3A and 3B) fit well with the theoretical functions (Eq. (1) and Eq. (2)) derived from the assumption of the binding of two cations per phosphorylation site. The fitting was achieved with the set of constants given in Table 1. The set of values indicates that the binding of phosphate slightly decreases the true affinity of the protein for at least the first Mn, as shown in Fig. 4, and predicts that the true affinity of the enzyme for P_i should decrease in the presence of Mn. The increase of the apparent affinities for P_i and Mn observed upon increasing [Mn] and [P_i] (Fig. 3C and 3D, respectively), is due to the favored formation of the covalent phosphoenzyme through step 5 of Scheme 1. The kinetic measurements of the fluorescence change show that the phosphorylation and dephosphorylation rate constants are 40 s^{-1} and 1 s^{-1}, respectively (Fig. 5). These values are comparable with those previously observed with magnesium [8]. The equilibrium constants of the reaction model (Table 1) were also applied to Eq. (3) and Eq. (4). The theoretical functions, drawn as continuous traces in Fig. 5B and 5C, also agree with the experimental values. The apparent affinity of Mn for the phosphorylation of the Ca-ATPase with P_i, in the presence of saturating P_i (Fig. 3D), is greater than that reported for Mg [8]. The binding of two Mn with P_i at the catalytic site may be important to stabilize the transition state of the phosphate [23] as previously described with several other proteins [24].

Acknowledgements

This work was supported by grants from the Universidad de Buenos Aires and from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

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