Cooperative calcium binding and calmodulin regulation in the calcium-dependent adenosine triphosphatase purified from the erythrocyte membrane

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Calcium binding to calcium-dependent ATPase purified from erythrocyte membrane was assessed by measurements of the ATPase intrinsic fluorescence. Calcium-binding isotherms obtained by fluorescence titration are identical to curves representing the Ca^{2+}-concentration dependence of ATPase activity, and demonstrate that cooperativity is in fact a feature of the binding mechanism rather than an apparent effect of enzyme kinetics. Loss of cooperativity and a reduction of the ATPase affinity for calcium is observed at very low enzyme concentrations. This effect of enzyme dilution is prevented by calmodulin at 37°C but not at 25°C. It is suggested that calcium binding by erythrocyte-membrane ATPase is influenced by hydrophobic interactions of binding domains, exhibiting a dissociation constant between 10^{-7} and 10^{-8} M in the absence of calmodulin, at 37°C and in a specific set of experimental conditions. The dissociation constant is decreased by calmodulin.

Erythrocyte membrane Ca^{2+}-ATPase   *Calmodulin*   *Purified Ca^{2+}-ATPase*   *Intrinsic fluorescence*
Cooperativity

1. INTRODUCTION

The ATP-driven calmodulin-sensitive calcium pump of the RCM maintains the cytosolic concentration of free Ca^{2+} below the micromolar level [1]. A calcium-stimulated ATPase which is the enzymatic expression of this pump, has been studied in RCM ghosts, in solubilized RCM and with enzyme purified from RCM [2–8]. A puzzling feature of this system is that the calcium-concentration dependence of ATPase activation and the effect of calmodulin are somewhat variable, evidently due to specific experimental features of different preparations. For instance, in RCM ghosts some variability is related to the

Abbreviations: RCM, erythrocyte membrane; SR, sarcoplasmic reticulum; $C_{12}E_8$, dodecyl octaethylene glycol monoether

degree of resealing and to residual calmodulin [5,9]. On the other hand, the purified enzyme is influenced by partial proteolysis [10,11] acidic phospholipids [11–13] and detergent [8,13,14]. It is not yet clear whether in the purified enzyme, calmodulin only increases the affinity of the ATPase for calcium or its maximal velocity or both. In fact, calcium binding to RCM ATPase was never measured at equilibrium, owing to its low stoichiometry. Therefore, the affinity of the enzyme for calcium has been evaluated indirectly from the calcium-concentration dependence of ATPase steady-state velocity. We have found that in analogy to the calcium ATPase of sarcoplasmic reticulum [15,16] the purified RCM ATPase undergoes a change of intrinsic fluorescence upon specific calcium binding. Thereby, it is possible to obtain equilibrium isotherms and transient signals related to calcium binding to the RCM ATPase. It

is also possible to demonstrate cooperative binding at equilibrium, matching the steady-state behavior of the ATPase in certain conditions. The cooperative character of the Ca^{2+}-concentration dependence of the RCM ATPase is lost upon dilution of the enzyme in detergent solutions, and such a loss is prevented by calmodulin through a temperature-dependent interaction with the enzyme.

2. MATERIALS AND METHODS

RCM Ca^{2+}-ATPase was prepared from human erythrocyte ghosts by calmodulin affinity chromatography by a method similar to that described by Niggli et al. [7] and Gietzen et al. [8]. The ghosts were solubilized in 0.4% $C_{12}E_8$, 10 mM Tris-maleate (pH 7.5), 130 mM KCl, 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 2 mM dithiothreitol and 20% glycerol, to a final concentration of 10 mg/ml. After a 10 min incubation in ice and centrifugation at 100000 × g for 40 min, the following protease inhibitors were added to the supernatant: 0.1 mM phenylmethanesulfonyl fluoride, 100 kallikrein units of aprotinin/ml and 0.02% thimerosal. 20 ml of this supernatant were loaded onto a 4 ml Sepharose 4B-calmodulin column equilibrated with the buffer used for solubilization. The loaded column was washed with 30 ml of the same buffer (including protease inhibitors) containing 0.04% $C_{12}E_8$ to remove the non-bound protein. The ATPase was eluted with buffer containing 0.04% $C_{12}E_8$, 5 mM EGTA and no calcium or protease inhibitors. Loading and elution flow was 0.25 ml/min, washing flow was 0.5 ml/min. The entire procedure was carried out in the cold room. The yield of purified protein was 0.12–0.14% of the ghost protein. Each ml of eluted enzyme was supplemented with 20 μl of a sonicated 1% suspension of egg-yolk phosphatidylcholine in elution medium, vortex-mixed and stored at −70°C. Electrophoretic analysis of this preparation displayed mostly one band of $M_r \simeq 140000$.

SR vesicles were prepared from rabbit skeletal muscle as described [17]. ATPase activity was determined by measurement of P_i production [18].

Steady-state intrinsic protein fluorescence was measured with an Aminco-Bowman spectrofluorometer equipped with a thermostated cell

holder and cuvette stirrer. The excitation wavelength was 290 nm and the emission 330 nm. Transient fluorescence changes were measured in a Dionex stopped-flow spectrofluorometer equipped with a 75 W mercury-doped xenon lamp. Excitation and emission wavelength were selected by interference and cutoff filters, respectively, to optimize tryptophan intrinsic fluorescence.

Free Ca^{2+} concentrations were estimated from total calcium and EGTA by computations [19] based on the Ca^{2+}-EGTA constant given by Schwartzenbach et al. [20] and corrected for pH, Mg concentration and nucleotide concentration, when present.
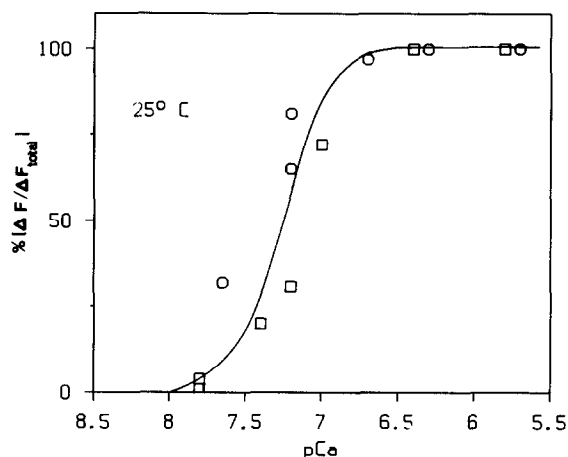

Fig.1. Change of RCM ATPase intrinsic fluorescence as a function of Ca^{2+} concentration. Reaction mixture: 100 mM Tris-chloride (pH 7.5), 0.15 mM $C_{12}E_8$, 130 mM KCl, 8 mM $MgCl_2$, 1 mM EGTA, 0.4 mM DTT and 0.01 mg RCM ATPase/ml. Sequential increments of free calcium (○) were obtained by adding small volumes of a concentrated $CaCl_2$ solution. Reverse titration was achieved by addition of small volumes of a concentrated EGTA solution (□) to ATPase saturated with calcium. The measurements were obtained at 25°C with an Aminco-Bowman spectrofluorometer, equipped with thermostatted cell holder and cuvette stirrer. The excitation wavelength was 290 nm and the emission wavelength was 330 nm. The experimental points were fitted with an iterative nonlinear regression method [24] according to

$$Vel = \frac{[Vel_{max}] [free\ Ca^{2+}]^n}{K + [free\ Ca^{2+}]^n}$$

where n is the Hill coefficient.

3. RESULTS

In analogy to the SR ATPase [15,16,21], we found that addition of micromolar free Ca^{2+} to RCM Ca^{2+}-ATPase preincubated with EGTA, is followed by an increase of intrinsic fluorescence (fig.1) up to a maximum of 3–4% of the fluorescence observed in the absence of calcium. Titration of the calcium-dependent fluorescence effect yields a highly cooperative isotherm, with a Hill coefficient = 2.7 and half-maximal saturation at 0.06 μM Ca^{2+}. The fluorescence enhancement can be readily reversed by stepwise addition of EGTA, yielding a curve identical to that obtained with calcium in the forward direction. A finding of considerable interest is that the fluorescence rise produced by addition of calcium to RCM ATPase is quite fast (fig.2A), while the rise obtained with SR vesicles (fig.2B) is much slower. The rapid rise is likely rendered possible by detergent exposure during the enzyme purification and removal of kinetic constraints related to the native membrane assembly of the enzyme. Analogous effects have been demonstrated for the SR ATPase [22].

It was originally shown that fluorometric calcium titration of SR ATPase generates curves which are identical to those obtained by direct measurement of calcium binding with radioactive calcium tracer, as well as to curves representing the Ca^{2+}-concentration dependence of ATPase activity [16,23]. Measurements of calcium binding to RCM ATPase were never performed because the radioactive tracer method requires amounts of protein which are much larger than the possible yield of purified RCM Ca^{2+}-ATPase. It can be demonstrated, however, that under the same experimental conditions the Ca^{2+}-concentration dependence of the fluorescence effect (fig.1) and that of RCM ATPase activity (fig.3) are identical. Therefore, we conclude that the fluorescence titration of the RCM Ca^{2+}-ATPase is representative of calcium binding to specific sites involved in enzyme activation.

One of the most interesting features of the RCM Ca^{2+}-ATPase is its stimulation by calmodulin. It is known, however, that calmodulin stimulation of the purified ATPase is somewhat variable, being influenced by partial proteolysis [10,11], the phospholipid composition of the reconstituted enzyme [11] and the concentration of detergents
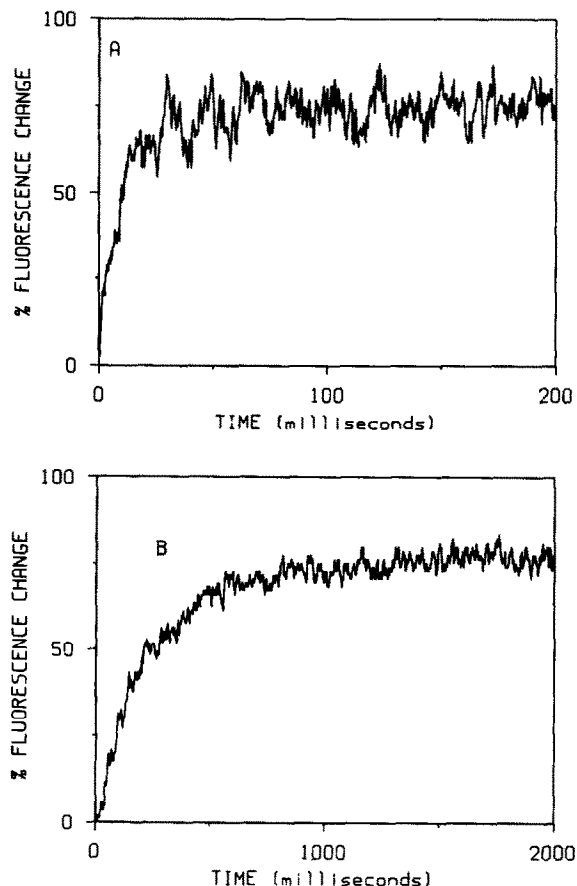

Fig.2. Intrinsic fluorescence change following rapid addition of calcium to RCM ATPase (A) and sarcoplasmic reticulum vesicles (B). The stopped-flow experiments were carried out with a Dionex apparatus. Note that the time scale is 200 ms for RCM ATPase (A) and 2000 ms for SR ATPase (B). The reaction mixture in syringe A contained 50 mM Tris-Cl (pH 7.5), 130 mM KCl, 8 mM $MgCl_2$, 1.3 mM EGTA, 0.5 mM $C_{12}E_8$, 14% glycerol, 1.4 mM DTT and 0.08 mg/ml RCM ATPase. The reaction mixture in syringe B contained the same concentration of Tris-Cl, KCl and $MgCl_2$ as in A and sufficient $CaCl_2$ to yield 0.05 mM free Ca^{2+}. The experiment with SR vesicles was carried out with a reaction mixture containing 50 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA and 0.1 mg SR per ml; $CaCl_2$ was added to yield 0.05 mM free Ca^{2+} as explained for the RCM ATPase. Temperature, 25°C.

[8,13]. In addition to these known variables, we found that the calmodulin effect is also dependent on the enzyme concentration in the assay medium, in the presence of a constant detergent concentra-
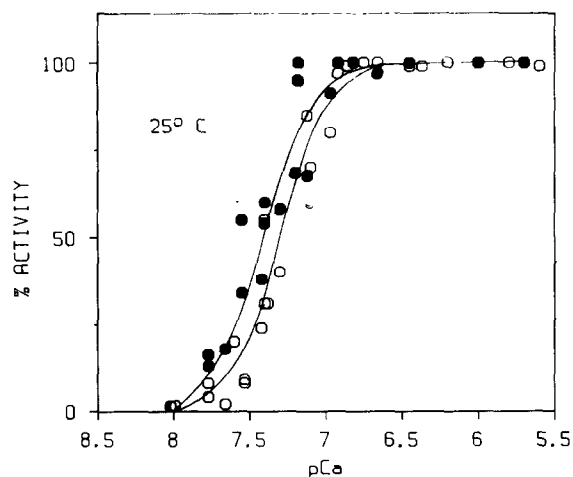
Fig.3. Ca^{2+}-concentration dependence of RCM ATPase at a relatively high enzyme concentration, in the presence or absence of calmodulin. Reaction mixture: 50 mM Tris-Cl (pH 7.5), 130 mM KCl, 8 mM $MgCl_2$, 1 mM EGTA, 0.15 mM $C_{12}E_8$, 4% glycerol, 0.4% DTT and 10 μg RCM ATPase per ml. Various amounts of $CaCl_2$ were added to yield the free Ca^{2+} concentration specified on the horizontal axis. Calmodulin, when present (●), was added at a 4:1 molar ratio with respect to the ATPase. The reaction was started by the addition of 3 mM ATP and carried out for 30 min at 25°C. The experimental points were fitted as in fig.1.

Fig.4. Effect of enzyme dilution, temperature and calmodulin on the Ca^{2+}-concentration dependence of RCM ATPase. The ATPase activity was measured in a reaction mixture identical to that described in fig.3, at 37°C (A) and 25°C (B), the reaction time was 30 and 60 min, respectively. The ATPase concentration was either 10 (○, ●) or 1 (△, ▲) μg/ml, in the absence (○, △) or presence (●, ▲) of calmodulin (calmodulin:enzyme molar ratio = 4).

tion. It is of interest, for instance, that at the concentration required to perform the fluorometric titration (10 μg/ml) we found no effect of calmodulin when either fluorescence (fig.1) or ATPase activity (fig.3) were measured. On the other hand, if the ATPase activity assay was repeated at lower enzyme concentrations (1 μg/ml), the Ca^{2+} dependence of ATPase activation in the absence of calmodulin displayed no cooperativity and was displaced toward higher Ca^{2+} concentrations (fig.4). When the reaction was carried out at 37°C, the dilution induced loss of cooperativity and reduction of calcium affinity were totally prevented by the presence of calmodulin (fig.4A). On the other hand, when the reaction was carried out at 25°C, the reduction of calcium affinity produced by enzyme dilution was much more pronounced and was not prevented by the presence of calmodulin (fig.4B).
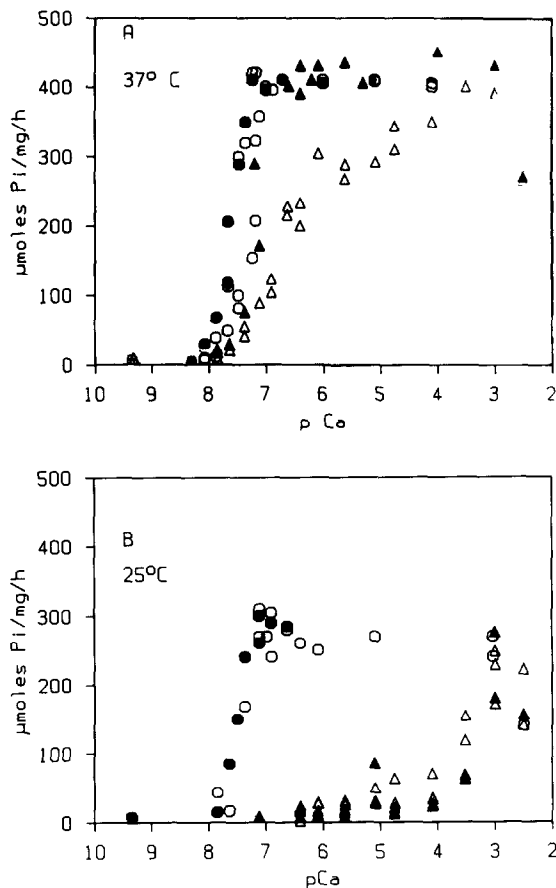
4. DISCUSSION

In the absence of direct measurements of calcium binding to the RCM ATPase, fluorometric titrations are a convenient method to assess the Ca^{2+}-concentration dependence of calcium binding at equilibrium, which can then be compared to the Ca^{2+} dependence of ATPase activation. It should be pointed out that even though the Ca^{2+} dependencies of the fluorescence effect and of en-

zyme activation are quite similar the fluorescence titration has the advantage of being amenable to equilibrium analysis, while the steady-state conditions of the enzyme activation are more difficult to interpret formally with respect to the binding mechanism. Furthermore, the fluorescence effect presents a practical advantage even over the direct determination of binding with radioactive tracer, inasmuch as it is suited to stopped-flow kinetic measurements. The identical Ca^{2+}-concentration dependencies of fluorescence effect and ATPase activity demonstrate that the fluorescence titration is related to calcium occupancy of specific sites involved in enzyme activation, and that the cooperativity of the Ca^{2+}-dependence curves is in fact a feature of the calcium binding mechanism rather than an apparent effect related only to enzyme kinetics.

Our findings that (a) the Ca^{2+}-concentration dependence of calcium binding and enzyme activation is highly cooperative and independent of calmodulin at higher enzyme concentrations, (b) cooperativity and calcium affinity are reduced at lower enzyme concentration, and (c) this effect of enzyme dilution is prevented by the presence of calmodulin at 37°C but not at 25°C, suggest that the calcium-binding mechanism is influenced by interaction of binding domains, and this in turn is facilitated by enzyme concentration and/or by the presence of calmodulin. The observed temperature dependence indicates the hydrophobic character of this interaction. If the interacting domains are assumed to be two ~140000 ATPase chains, these findings are consistent with a dissociation constant for the monomer/dimer equilibrium between 10^{-7} and 10^{-8} M in our reaction medium and at 37°C. The dissociation constant is decreased in the presence of calmodulin. It is likely that in the native erythrocyte membrane, the solvent characteristics of the lipid matrix and their influence on the ATPase interaction, establish the requirement for calmodulin regulation.

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