Characterization of Ruthenium Red-binding sites of the Ca^{2+} -ATPase from sarcoplasmic reticulum and their interaction with Ca^{2+} -binding sites

Senena CORBALAN-GARCIA, José A. TERUEL* and Juan C. GOMEZ-FERNANDEZ

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, Murcia, Spain

Sarcoplasmic reticulum Ca^{2+} -ATPase has previously been shown to bind and dissociate two Ca^{2+} ions in a sequential mode. This behaviour is confirmed here by inducing sequential Ca^{2+} dissociation with Ruthenium Red. Ruthenium Red binds to sarcoplasmic reticulum vesicles (6 nmol/mg) with a $K_d = 2 \mu\text{M}$, producing biphasic kinetics of Ca^{2+} dissociation from the Ca^{2+} -ATPase, decreasing the affinity for Ca^{2+} binding. Studies on the effect of Ca^{2+} on Ruthenium Red binding indicate that Ruthenium Red does not bind to the high-affinity Ca^{2+} -binding sites, as suggested by the following observations: (i) micromolar concentrations of Ca^{2+} do not significantly alter Ruthenium Red binding to the sarcoplasmic reticulum; (ii) quenching of the fluorescence of fluorescein 5'-isothiocyanate (FITC) bound to Ca^{2+} -ATPase by Ruthenium Red (resembling Ruthenium Red binding) is not prevented by micromolar concentrations of Ca^{2+} ; (iii) quenching of FITC fluorescence by Ca^{2+} binding to the high-affinity sites is achieved even though Ruthenium Red is bound to the Ca^{2+} -ATPase; and (iv) micromolar Ca^{2+} concentrations prevent inhibition of the ATP-hydrolytic capability by dicyclohexylcarbodi-imide modification, but Ruthenium Red does not. However, micromolar concentrations of lanthanides (La^{3+} and Tb^{3+}) and millimolar concentrations of bivalent cations (Ca^{2+} and Mg^{2+}) inhibit Ruthenium Red binding as well as quenching of FITC-labelled Ca^{2+} -ATPase fluorescence by Ruthenium Red. Studies of Ruthenium Red binding to tryptic fragments of Ca^{2+} -ATPase, as demonstrated by ligand blotting, indicate that Ruthenium Red does not bind to the A_1 subfragment. Our observations suggest that Ruthenium Red might bind to a cation-binding site in Ca^{2+} -ATPase inducing fast release of the last bound Ca^{2+} by interactions between the sites.

INTRODUCTION

Sarcoplasmic reticulum (SR) regulates Ca^{2+} distribution inside the muscle cell allowing contraction and relaxation [see Inesi *et al.* (1990) for a review]. Ca^{2+} -ATPase is the most abundant protein of isolated SR vesicles. It pumps Ca^{2+} from the cytoplasm of the muscle cell to the lumen of the SR, bringing about muscle relaxation. When Ca^{2+} -ATPase is working, ATP is hydrolysed and Ca^{2+} is accumulated inside the SR vesicles, maintaining a Ca^{2+} concentration gradient across the membrane. Ca^{2+} binding to Ca^{2+} -ATPase is an absolute requisite for enzyme activation, which allows phosphorylation by ATP followed by Ca^{2+} transport. Two high-affinity Ca^{2+} -binding sites face the cytoplasmic portion of the SR membranes, exhibiting positive co-operativity. From kinetic experiments, these two Ca^{2+} -binding sites, one easily accessible and a second less accessible site (Dupont, 1982; Nakamura, 1986; Inesi, 1987; Petithory & Jencks, 1988), can be resolved. It is proposed that these sites are located in helical segments clustered in the transmembrane domain of Ca^{2+} -ATPase, which may form a Ca^{2+} channel, the two sites being located in single file within a protein crevice. This model was first suggested by MacLennan *et al.* (1985) and points to a sequential mechanism for Ca^{2+} binding to Ca^{2+} -ATPase.

The spectral characteristics of lanthanides have been useful in structural studies on Ca^{2+} -ATPase (Stephens & Grisham, 1979; Highsmith & Head, 1983; Squier *et al.*, 1990). Lanthanides displace Ca^{2+} from specific sites in Ca^{2+} -ATPase with biphasic kinetics, suggesting sequential exchange; the sites are located midway between the catalytic site outside the membrane

and the transmembrane Ca^{2+} -binding domain (Squier *et al.*, 1990).

It is well accepted that Ruthenium Red is a powerful inhibitor of Ca^{2+} transport in mitochondria (Moore, 1971; Vasington *et al.*, 1972; Reed & Bygrave, 1974), in erythrocyte membranes (Watson *et al.*, 1971) and in SR of rabbit skeletal muscle (Vale & Carvalho, 1973; Madeira & Antunes-Madeira, 1974). Reed & Bygrave (1974) used Ruthenium Red and lanthanides as tightly binding inhibitors of Ca^{2+} transport in mitochondria. They found that the inhibition by Ruthenium Red is competitive for lanthanides but not for Ca^{2+} , and this is attributed to a second type of binding site for lanthanides which is different from the Ca^{2+} -transport site.

Ruthenium Red has also been reported to block Ca^{2+} -induced Ca^{2+} release from both skinned slow or fast-twitch skeletal and cardiac muscle fibres as well as SR vesicles from either skeletal or cardiac-muscle tissue (Chamberlain *et al.*, 1984; Volpe *et al.*, 1986). Ruthenium Red also binds to several Ca^{2+} -binding proteins, such as calsequestrin, Ca^{2+} -ATPase, erythrocyte Band 3 anion transporter, calmodulin, troponin C and S-100 (Charuk *et al.*, 1990).

Since Ruthenium Red appears to inhibit Ca^{2+} -dependent processes specifically, we have used this compound as a probe for the Ca^{2+} -binding sites of Ca^{2+} -ATPase. We find that the study of the interaction of Ca^{2+} and Ruthenium Red-binding sites provides a powerful tool for the analysis of the mechanism of Ca^{2+} binding as well as for localization of cation-binding sites in Ca^{2+} -ATPase, as Ruthenium Red interferes with the Ca^{2+} -binding mechanism.

Abbreviations used: C_{12}E_8 , dodecyl octaethylene glycol monoether; DCCD, dicyclohexylcarbodi-imide; FITC, fluorescein 5'-isothiocyanate; NCD4, *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodi-imide; SR, sarcoplasmic reticulum.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Dicyclohexylcarbodi-imide (DCCD), LaCl_3 , TbCl_3 and Ruthenium Red were obtained from Merck. Ruthenium Red was used after further purification by the procedure of Fletcher *et al.* (1961). The absorption spectrum of purified Ruthenium Red, $[(\text{NH}_3)_5\text{Ru}-\text{O}-\text{Ru}(\text{NH}_3)_4-\text{O}-\text{Ru}(\text{NH}_3)_5]^{6+}\text{Cl}_6\cdot 4\text{H}_2\text{O}$, showed a single band centred at 533 nm, with a molar absorption coefficient of $65000\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 533 nm, for an M_r of 858.3 according to the original literature (Fletcher *et al.*, 1961; Luft, 1971). The Ca^{2+} ionophore, A23187, was from Boehringer-Mannheim. Chemicals used in electrophoresis were purchased from Bio-Rad. Fluorescein 5'-isothiocyanate (FITC), bovine trypsin and trypsin inhibitor were obtained from Sigma. $^{45}\text{CaCl}_2$ was purchased from New England Nuclear. Dodecyl octaethylene glycol monoether (C_{12}E_8) was obtained from Nikko Chemicals C.O. (Tokyo, Japan). All other reagents were of analytical grade from Merck.

Membrane preparations

Native SR vesicles, mainly derived from longitudinal tubules of rabbit fast-twitch skeletal muscle, were prepared as described by Eletr & Inesi (1972). ATPase was solubilized with $1.5\text{ mM}-\text{C}_{12}\text{E}_8$ by the procedure of Kosk-Kosicka *et al.* (1983). In this membrane preparation, the content of ATPase is about 85% of total protein, and calsequestrin and the high-affinity Ca^{2+} -binding protein represent about 10% of the protein as estimated by densitometry of electrophoresis gels.

Ruthenium Red binding

Ruthenium Red binding to SR vesicles was assayed by incubating Ruthenium Red with SR vesicles in a medium containing 0.5 mg of protein/ml, 1 mM-EGTA, 20 mM-Mops, pH 6.8, 80 mM-KCl and various concentrations of Ruthenium Red. After centrifuging at 74000 g for 40 min at 25°C , the pellet was resuspended in 1% SDS, and the absorbance was measured at 533 nm for Ruthenium Red determination. A calibration curve with standard Ruthenium Red showed that, within the range of concentration used, there is a linear relationship between concentration and absorbance. However, we find that it is difficult to calculate bound Ruthenium Red by difference between total and free Ruthenium Red because there is a significant non-specific binding to glass, filters, etc., and it becomes very dependent on the experiment we are carrying out. Therefore we have to measure directly bound and free Ruthenium Red.

Ca^{2+} binding

The equilibrium of Ca^{2+} binding to Ca^{2+} -ATPase was measured by incubating 0.32 mg of SR vesicles/ml in a buffer containing 20 mM-Mops, pH 6.8, 80 mM-KCl, 100 μM -Ruthenium Red, 100 $\mu\text{M}-^{45}\text{Ca}^{2+}$ and various EGTA concentrations to reach the desired value of pCa. After 15 min of incubation, a portion of 0.16 mg of protein was filtered through Millipore filters (HAWP 0.45 μm pore size). The ^{45}Ca retained in the filter was determined by liquid-scintillation counting. The blanks were obtained including 10 mM- LaCl_3 in the incubation medium, being about 0.5 nmol of bound Ca^{2+} per mg of SR vesicles.

The kinetics of Ca^{2+} dissociation were measured with the aid of a rapid-filtration apparatus (Bio-Logic, Claix, France), using DAWP 0.65 μm Millipore filters. SR vesicles (80 μg) pre-incubated in a buffer containing 20 mM-Mops, pH 6.8, 80 mM-KCl and 5 $\mu\text{M}-^{45}\text{CaCl}_2$ were placed on a filter, and then perfused with a water solution of Ruthenium Red for time-intervals ranging from 20 ms to 10 s. Thereafter, the filters were collected for ^{45}Ca determination. The kinetics of Ca^{2+} binding were assayed

by perfusing SR vesicles on the filter with a solution containing Ruthenium Red and $^{45}\text{CaCl}_2$ in a buffer containing 20 mM-Mops, pH 6.8, and 80 mM-KCl.

Trypsin digestion

Tryptic digestion of SR vesicles was carried out at 25°C in 20 mM-Mops (pH 6.8)/80 mM-KCl and trypsin at a trypsin/SR ratio up to 1:2 (w/w) for 10 min. The reaction was stopped by adding soya-bean trypsin inhibitor at a concentration twice as high as that of trypsin.

Electrophoresis and blotting

SDS/PAGE of intact and digested SR vesicles was performed as described by Laemmli (1970), using 10–19% (w/v) polyacrylamide gradient minigels. Electrophoretic transfer of SR proteins from minigels to nitrocellulose papers was carried out in 25 mM-Tris/192 mM-glycine, pH 8.3, buffer, using 100 V and 0.7 A constant current for 5 h. Gels were stained with Coomassie Blue R250 and nitrocellulose papers were pre-equilibrated with 10 mM-Tris/HCl (pH 7.5)/80 mM-KCl buffer for 30 min, and then incubated in the same solution containing 25 μM -Ruthenium Red for the Ruthenium Red-binding assay. Proteins on nitrocellulose were stained with Amido Black.

FITC and DCCD labelling

SR vesicles were labelled with FITC as described previously (Teruel & Inesi, 1988), and with 0.2 mM-DCCD essentially as described by de Ancos & Inesi (1988), including various concentrations of Ca^{2+} or Ruthenium Red in the reaction medium.

Fluorescence measurements

Fluorescence measurements were performed in a Shimadzu RF-540 spectrofluorimeter equipped with thermostatically controlled cells and magnetic stirrer. FITC-labelled SR-vesicle fluorescence emission was recorded at 520 nm (exciting wavelength 480 nm).

Assays

ATPase activity was assayed by a colorimetric determination of inorganic phosphate as described by Lin & Morales (1977). Protein concentration was estimated by the procedure of Lowry *et al.* (1951), with BSA as standard. Free Ca^{2+} concentration was estimated from total Ca^{2+} and EGTA concentrations (Fabiato & Fabiato, 1979), taking into account pH, Mg^{2+} , K^+ , P_i and nucleotide concentrations.

All data shown in the Figures were from experiments that were repeated at least three times. In the cases where error bars are not shown the data correspond to representative experiments.

RESULTS

Ca^{2+} binding

The equilibrium of Ca^{2+} binding was studied by incubating SR vesicles with ^{45}Ca in a Ca^{2+} -buffered medium. Ca^{2+} binding was altered by the presence of micromolar Ruthenium Red concentrations, which displaced the titration curve to higher Ca^{2+} concentrations, but the same maximum of Ca^{2+} binding was reached in the absence and presence of Ruthenium Red (Fig. 1a). This behaviour would be expected if Ruthenium Red binds to the high-affinity Ca^{2+} -binding site, the observed dissociation constant (K_d) being higher and Ruthenium Red being displaced by high Ca^{2+} concentration, indicating competitive inhibition. However, the same behaviour would also be expected if Ruthenium Red binds to other sites and modifies the Ca^{2+} -binding site, decreasing the affinity for Ca^{2+} but not the total number of bound Ca^{2+} ions,

Fig. 1. Equilibrium of Ca^{2+} binding

(a) SR vesicles (0.32 mg/ml) were incubated with Ca^{2+} in the absence (●) or presence (○) of 100 μM -Ruthenium Red in 20 mM-Mops (pH 6.8)/80 mM-KCl/5 mM-MgCl₂/100 μM - $^{45}\text{CaCl}_2$ containing various EGTA concentrations to establish different values of free $[\text{Ca}^{2+}]$. (b) SR vesicles (0.08 mg/ml) were incubated in a medium containing 20 mM-Mops, pH 6.8, 80 mM-KCl, 10 μM - $^{45}\text{CaCl}_2$ and different concentrations of Ruthenium Red. After 15 min of incubation at room temperature, portions of 0.5 ml (a) or 1 ml (b) were filtered and ^{45}Ca was counted for radioactivity. Blanks were performed which included 10 mM-LaCl₃ in the incubation medium. Error bars are standard deviations from the mean for three experiments.

indicating non-competitive inhibition. Therefore from this experiment it cannot be concluded whether Ruthenium Red acts as a competitive inhibitor of Ca^{2+} binding or not. The equilibrium of Ca^{2+} binding was also studied by changes in the quantum yield of tryptophan residues of Ca^{2+} -ATPase promoted by free Ca^{2+} in the medium. In this case, the same behaviour was observed by measuring the increments in intrinsic fluorescence induced by Ca^{2+} (results not shown). The binding curves were fitted to the Hill equation, the Hill number being 1.5 and 0.94 in the absence and presence of Ruthenium Red respectively. Ruthenium Red produced a loss of co-operativity in Ca^{2+} binding and also a decrease in the affinity for Ca^{2+} binding.

Ca^{2+} binding was also studied at different Ruthenium Red concentrations (Fig. 1b). In this experiment, SR vesicles were incubated with 10 μM - $^{45}\text{Ca}^{2+}$ and various Ruthenium Red concentrations for 15 min before being filtered for ^{45}Ca counting. The titration curve of Ca^{2+} binding showed two well-differentiated saturating effects, which represent two distinct Ca^{2+} -binding sites made apparent at different Ruthenium Red concentrations. This curve fits to two-sigmoid equation where the Hill number is 1.0, and the K_m is 4.5 μM and 2.0 mM for the two Ca^{2+} -binding sites. At low Ruthenium Red concentration, half of the bound Ca^{2+} is released, i.e. one Ca^{2+} ion per active site of Ca^{2+} -ATPase, and the remaining bound Ca^{2+} needs millimolar Ruthenium Red concentrations to be dissociated from the protein, at the free Ca^{2+} concentration used in this experiment. The fact that Ruthenium Red can differentiate between two Ca^{2+} ions bound to the high-affinity Ca^{2+} -binding sites means that they are not identical binding sites, Ca^{2+} being displaced by Ruthenium Red from the first site more easily than from the second.

Since Ruthenium Red modifies the equilibrium constant for Ca^{2+} binding, we studied the effect on the component steps: the kinetics of Ca^{2+} dissociation and binding. These experiments were performed with the aid of a rapid-filtration apparatus. For Ca^{2+} dissociation, the perfusion medium was a water solution of Ruthenium Red to ensure a low Ca^{2+} content in the medium. The amount of Ca^{2+} in our deionized water was always below 1 μM , as detected by atomic absorption spectrometry. This low Ca^{2+} concentration did not induce Ca^{2+} exchange in our experimental conditions (Fig. 2). When the filter containing SR vesicles with

Fig. 2. Kinetics of Ca^{2+} dissociation

SR vesicles (0.08 mg/ml) were incubated in a medium containing 20 mM-Mops, pH 6.8, 80 mM-KCl, 5 mM-MgCl₂ and 5 μM - $^{45}\text{CaCl}_2$. Portions of 1 ml were filtered and washed with 1 ml of water. Then the filters were perfused at various time-intervals with water (○) or 100 μM -Ruthenium Red (●) (a), or with water (○), 1 mM- $^{40}\text{CaCl}_2$ (□), 100 μM -Ruthenium Red (●) or 10 mM-EGTA (△) (b).

Fig. 3. Kinetics of Ca^{2+} association

SR vesicles (0.08 mg/ml) in 20 mM-Mops/80 mM-KCl/5 mM-MgCl₂/1 mM-EGTA were filtered, and the filters were washed with 3 ml of water. They were then perfused at various time-intervals with the same buffer containing 5 μM - $^{45}\text{CaCl}_2$ and various Ruthenium Red concentrations: no Ruthenium Red (○), 0.5 μM (●), 5 μM (△) and 100 μM (▲). Error bars are standard deviations from the mean for three experiments.

bound Ca^{2+} was perfused with 100 μM -Ruthenium Red, very rapid Ca^{2+} dissociation was observed, which corresponds to only one Ca^{2+} ion per active site of the Ca^{2+} -ATPase (Fig. 2a). The second Ca^{2+} ion was also released, but over a longer time-scale. In the same experimental conditions, EGTA produced complete dissociation of Ca^{2+} , and millimolar concentrations of $^{40}\text{Ca}^{2+}$ produced a fast exchange with $^{45}\text{Ca}^{2+}$ at only one Ca^{2+} site (Fig. 2b).

The effect of Ruthenium Red on Ca^{2+} association was also studied. SR vesicles were layered on a filter, and then perfused with Ca^{2+} plus Ruthenium Red. The ability of SR vesicles to bind Ca^{2+} was decreased by micromolar Ruthenium Red concentrations (Fig. 3). At 100 μM -Ruthenium Red, no Ca^{2+} binding was observed during 1 s of perfusion time, with 5 μM - Ca^{2+} added to the medium.

Ruthenium Red binding

Ruthenium Red bound to SR vesicles was measured after the separation of bound and free Ruthenium Red by ultracentrifugation. The pellets of SR vesicles with bound Ruthenium Red were redissolved in an SDS-containing buffer, and the Ruthenium Red content was determined spectroscopically at 533 nm (Fig. 4). The Ruthenium Red-binding curve reached saturation at about 6 nmol of Ruthenium Red per mg of protein,

Fig. 4. Binding of Ruthenium Red to SR vesicles

SR vesicles (0.5 mg/ml) were incubated in 20 mM-Mops (pH 6.8)/80 mM-NaCl containing various Ruthenium Red concentrations to achieve the free concentrations shown and 1 mM-EGTA (●) or 0.1 mM- CaCl_2 (○). Free and bound Ruthenium Red were separated by spinning down the SR vesicles, and the pellets were assayed for bound Ruthenium Red.

which corresponds to approximately one Ruthenium Red molecule bound per active site of Ca^{2+} -ATPase, assuming four to six catalytic sites per mg of protein, and shows a K_d of 2 μM . When 0.1 mM-free Ca^{2+} was included in the medium, the same Ruthenium Red-binding curve was obtained. Therefore this experiment indicates that occupancy of high-affinity Ca^{2+} -binding sites does not significantly alter Ruthenium Red binding to SR.

Fig. 5 shows the effect of metal ions (La^{3+} , Tb^{3+} , Ca^{2+} and Mg^{2+}) on Ruthenium Red binding to SR vesicles. The SR vesicles were incubated with 5 μM -Ruthenium Red and different concentrations of metal. Lanthanides (La^{3+} and Tb^{3+}) inhibited binding of Ruthenium Red at micromolar concentrations (Fig. 5a). However, millimolar concentrations of bivalent cations, such as Ca^{2+} and Mg^{2+} , were needed to inhibit Ruthenium Red binding (Fig. 5b). From the above, it is clear that Ruthenium Red binds to a part of the protein at which these metals also bind, but which is different from the high-affinity Ca^{2+} -binding sites, although they are affected by Ruthenium Red (Fig. 1).

In order to elucidate where the Ruthenium Red-binding sites are located, we digested Ca^{2+} -ATPase with trypsin at a trypsin/ Ca^{2+} -ATPase ratio of 1:2 (w/w) to produce controlled splitting of Ca^{2+} -ATPase molecules at the T_1 site (Arg-505; MacLennan *et al.*, 1985; Brandl *et al.*, 1986) yielding two fragments, A and B, and also splitting at the T_2 site (Arg-198; MacLennan *et al.*, 1985; Brandl *et al.*, 1986) yielding subfragments A_1 (M_r 33270) and A_2 (M_r 22115) from fragment A. These fragments were separated by SDS/PAGE in a gradient of polyacrylamide, then electrotransferred to nitrocellulose paper on which Ruthenium Red binding was assayed. The fragments with bound ligand were

Fig. 5. Effect of cations on Ruthenium Red binding to SR vesicles

SR vesicles (0.5 mg/ml) were incubated in 20 mM-Mops (pH 6.8)/80 mM-NaCl/5 μM -Ruthenium Red containing LaCl_3 (●) or TbCl_3 (○) (a), or CaCl_2 (●) or MgCl_2 (○) (b).

seen by direct staining with Ruthenium Red. It was found that Ruthenium Red binds to Ca^{2+} -ATPase (M_r 110000) as well as to other proteins, such as calsequestrin (M_r 40000) and an intrinsic glycoprotein (M_r 160000) (results not shown). In fragmented Ca^{2+} -ATPase, Ruthenium Red binds to the A_2 subfragment but not to the A_1 subfragment.

FITC labelling

Ca^{2+} -ATPase was covalently labelled with FITC in order to produce a probe located at or close to the nucleotide-binding site. The fluorescence emission spectrum of FITC bound to SR, on excitation at 480 nm, has an emission maximum centred at 520 nm. The energy emitted by FITC can be reabsorbed by Ruthenium Red, since its absorbance spectrum has a maximum centred at 533 nm. Thus we can predict quenching of FITC fluorescence in the presence of Ruthenium Red by Förster fluorescence energy transfer. Fig. 6 shows the quenching of FITC fluorescence at various Ruthenium Red concentrations. In our experimental conditions, quenching of the fluorescence of FITC in solution was negligible. The total lack of absorbance of Ruthenium Red in the region of the spectrum where FITC absorbs light rules out an internal filter effect. This curve represents binding of Ruthenium Red to the protein and therefore demonstrates the proximity of Ruthenium Red to the FITC label. Furthermore, the concentration range of Ruthenium Red necessary for FITC quenching is the same as for binding of Ruthenium Red to the protein (compare Figs. 4 and 6). This means that, at these concentrations of Ruthenium Red, two processes take place: binding of Ruthenium Red to its binding sites in the protein (Fig. 4) and the transfer of energy from the

Fig. 6. Quenching of FITC fluorescence by Ruthenium Red

Fluorescence of FITC-labelled SR (0.06 mg/ml) (●) and FITC-labelled ATPase solubilized with C_{12}E_8 (○) was quenched with Ruthenium Red in a buffer containing 20 mM-Mops, pH 6.8, 80 mM-KCl, 0.1 mM-EGTA and 0.2 mM- CaCl_2 . FITC label was excited at 480 nm and its emission fluorescence was recorded at 520 nm.

Fig. 7. Effect of cations on the quenching of FITC-labelled SR fluorescence by Ruthenium Red

FITC-labelled SR fluorescence was quenched by addition of 0.4 μM -Ruthenium Red. Then, 'dequenching' was achieved after addition of (a) LaCl_3 (○) or TbCl_3 (●), or (b) MgCl_2 (○) or CaCl_2 (●). F_0 represents the fluorescence quenched by Ruthenium Red.

Fig. 8. Titration of FITC-labelled SR fluorescence with Ca^{2+}

FITC-labelled SR (0.06 mg) fluorescence was quenched by addition of $0.4 \mu\text{M}$ -Ruthenium Red, and then increasing concentrations of Ca^{2+} were added. A scheme of an experimental record is drawn in the inset.

FITC site to the Ruthenium Red site (Fig. 6). The same behaviour is also demonstrated for solubilized ATPase labelled with FITC. In this case also there is quenching of FITC fluorescence (Fig. 6, open circles) which demonstrates direct binding of Ruthenium Red to the protein, with no significant binding to lipids. However, a slight decrease in the affinity for Ruthenium Red binding is observed in solubilized ATPase with respect to native SR vesicles. In order to corroborate the finding that quenching of FITC fluorescence is due to Ruthenium Red binding, we induced 'dequenching' of FITC fluorescence by the addition of cations, which inhibit Ruthenium Red binding, as previously demonstrated in this work (Fig. 5). These cations (La^{3+} , Tb^{3+} , Ca^{2+} and Mg^{2+}) promoted the recovery of FITC fluorescence in the presence of Ruthenium Red (Fig. 7). The inhibition of Ruthenium Red binding by these cations completely restored the initial FITC fluorescence. In addition, the concentrations of La^{3+} and Tb^{3+} that inhibited Ruthenium Red quenching were in the micromolar range (Fig. 7a), whereas Ca^{2+} and Mg^{2+} inhibited the same process in the millimolar range (Fig. 7b). These are the same concentration ranges as observed for the inhibition of Ruthenium Red binding by La^{3+} and Tb^{3+} (Fig. 5a) and Ca^{2+} and Mg^{2+} (Fig. 5b). The same result was obtained with solubilized ATPase (results not shown), indicating that, at micromolar concentrations, Ruthenium Red binds specifically to Ca^{2+} -ATPase, and binding to lipids can be excluded. In parallel experiments, we checked that these cations did not modify the quantum yield of FITC in solution, in the concentration range under study. Since all the fluorescence is restored by inhibition of Ruthenium Red binding, we can assume that collisional quenching produced by Ruthenium Red is negligible. Therefore the quenching of FITC fluorescence in solution by Ruthenium Red can be attributed mostly to Förster energy transfer from FITC to free Ruthenium Red.

FITC bound to Ca^{2+} -ATPase has been extensively used as a probe to monitor Ca^{2+} -binding to its high-affinity binding sites, since FITC quantum yield decreases about 10% on Ca^{2+} binding (Pick & Karlsh, 1980). In order to check whether Ruthenium

Fig. 9. Effect of Ruthenium Red and Ca^{2+} on DCCD labelling

SR vesicles (1 mg/ml) were incubated in the dark for 2 h at 4°C in a medium containing 50 mM-Mes, pH 6.0, 100 mM-KCl, $40 \mu\text{M}$ -A23187, 0.2 mM-DCCD and increasing concentrations of CaCl_2 (▲) or Ruthenium Red (●). To stop the reaction, samples (0.06 ml) were diluted in 3 ml of a medium containing 50 mM-Mops, pH 6.8, 80 mM-KCl, 1 mM-EGTA, 1 mM- CaCl_2 , $10 \mu\text{M}$ -A23187 and 1 mM-ATP, in which ATPase activity was assayed. A control experiment was performed under the same conditions but in the absence of DCCD (○).

Red is bound to these high-affinity Ca^{2+} -binding sites or not, we studied the emission fluorescence of FITC in the presence of $2 \mu\text{M}$ -Ruthenium Red, which produces approximately 40% quenching at low micromolar Ca^{2+} concentration. We found that the addition of micromolar concentrations of Ca^{2+} quenched the FITC fluorescence even in the presence of Ruthenium Red (Fig. 8), which is the opposite of what we would expect if there was competition between the two sites. The Ruthenium Red- and Ca^{2+} -binding sites can be titrated by FITC quenching at the same time. Thereafter, the addition of millimolar concentrations of Ca^{2+} 'dequenched' the FITC fluorescence previously quenched by Ruthenium Red, which resembles the inhibition of Ruthenium Red binding by high Ca^{2+} concentrations (an experimental record is shown in the inset of Fig. 8).

DCCD labelling

DCCD is widely used to modify carboxy groups, such as aspartate and glutamate residues in proteins. At low concentrations it reacts with an acidic residue of the Ca^{2+} -ATPase involved in Ca^{2+} binding (Pick & Racker, 1979; Murphy, 1981; Scofano *et al.*, 1985). Furthermore, a fluorescent derivative has also been used as a probe to locate the high-affinity Ca^{2+} -binding sites for structural analysis (Chadwick & Thomas, 1983, 1984; Pick & Weiss, 1985; Munkonge *et al.*, 1989). In this work we have used DCCD to modify the environment of the high-affinity Ca^{2+} -binding sites, and to study the effect of Ruthenium Red binding on the Ca^{2+} -binding sites. The effect of DCCD labelling can be monitored by measuring ATP hydrolysis, after preincubating the Ca^{2+} -ATPase with DCCD. Preincubation with 0.2 mM-DCCD for 2 h abolished almost completely the ATP-hydrolytic capability of Ca^{2+} -ATPase (Fig. 9), which has been attributed to inhibition of Ca^{2+} binding (Pick & Racker, 1979; Murphy, 1981; Scofano *et al.*, 1985). Therefore preincubation in the presence of micromolar concentrations of Ca^{2+} prevented DCCD labelling of the high-affinity binding sites. However, when the preincubation was carried out in the presence of Ruthenium Red, protein modification by DCCD was not prevented up to $100 \mu\text{M}$ -Ruthenium Red (Fig. 9).

DISCUSSION

The effect of Ruthenium Red on the equilibrium of Ca^{2+} binding (Fig. 1a) may be interpreted in terms of competition between Ruthenium Red- and Ca^{2+} -binding sites and also in

DCCD labelling inhibits the ATPase activity by blocking Ca^{2+} binding, because DCCD modifies some amino acid residues involved in the Ca^{2+} -binding domain. The inhibition by DCCD can be prevented by the presence of Ca^{2+} in the reaction medium (Fig. 9). However, Ruthenium Red does not interfere with DCCD labelling. This experiment indicates that Ruthenium Red binds to amino acid residues not involved in DCCD labelling and, by extension, not directly involved in Ca^{2+} binding. Thus the Ruthenium Red-binding domain and DCCD-labelled residue are not identical, despite the fact that both are located in the same tryptic subfragment. This result supports other experimental evidence provided here that the two sites, i.e. Ruthenium Red- and Ca^{2+} -binding sites, are not identical, although they might be proximal. Recently, the peptide segment at which *N*-cyclohexyl-*N'*-(4-dimethylamino-1-naphthyl)carbodi-imide (NCD4) reacts has been identified: the NCD4 label resides in subfragment A₁ between Glu-231 and Glu-309, and this labelling is prevented by Ca^{2+} (Sumbilla *et al.*, 1991). Mutations of other amino acid residues more distant in the primary structure have been proposed to interfere with Ca^{2+} binding. These residues are between Glu-771 and Glu-908 in the B tryptic fragment (Clarke *et al.*, 1989), probably located in helical segments in the transmembrane region. Thus protein folding might allow formation of an ionic transmembrane channel in which mutant amino acid residues from different regions of the primary structure might participate.

We have shown that titration of the high-affinity Ca^{2+} -binding sites can be achieved, even though Ruthenium Red remains bound to the SR, and occupancy of these sites does not interfere with FITC-labelled SR quenching by bound Ruthenium Red (Fig. 8). However, micromolar concentrations of lanthanides and millimolar concentrations of Ca^{2+} and Mg^{2+} caused Ruthenium Red dissociation and 'dequenched' the FITC-labelled SR fluorescence previously quenched by Ruthenium Red (Figs. 5, 7 and 8). These experiments demonstrate that the high-affinity Ca^{2+} -binding sites are different from the Ruthenium Red-binding sites, and that Ruthenium Red may bind to a cation-binding site in Ca^{2+} -ATPase proposed to be in the stalk portion of the enzyme [model of MacLennan *et al.* (1985)] which would contain several acidic residues with cation-binding ability (Brandl *et al.*, 1986). On the other hand, it has been reported that the presence of millimolar concentrations of Mg^{2+} alters the kinetics of Ca^{2+} binding to Ca^{2+} -ATPase, which has been attributed to formation of a stabilized form of this enzyme (Guillain *et al.*, 1982; Champeil *et al.*, 1985). More recently it has been attributed to binding of Mg^{2+} to the outer Ca^{2+} site, inducing a rapid Ca^{2+} - Mg^{2+} exchange, hence accelerating binding of the first Ca^{2+} (Mountin & Dupont, 1990). Highsmith & Head (1983) pointed out that lanthanides may bind to the Mg^{2+} -binding site in the 10–100 μM concentration range, and this site is located near the FITC label [approx. 1 nm (10 Å)] in the nucleotide-binding domain of Ca^{2+} -ATPase (Highsmith & Head, 1983; Highsmith, 1984).

It has been reported that Ruthenium Red inhibits phosphorylation by inorganic phosphate by competing with Mg^{2+} (de Meis, 1991; de Meis *et al.*, 1991). Therefore Ruthenium Red might also bind to the Mg^{2+} -binding site of Ca^{2+} -ATPase where Ca^{2+} (at millimolar concentration) and lanthanides (at micromolar concentration) might also bind, or it binds to a non-specific binding site located in the stalk portion of the protein.

In conclusion, the Ruthenium Red-binding site might be a cation-binding site at which bivalent cations bind with low affinity and lanthanides and Ruthenium Red bind with high affinity. This site is probably located in the stalk portion of the protein near the Ca^{2+} -binding site, allowing interactions between the two sites, which result in rapid Ca^{2+} dissociation.

This work was supported by grants no. PB87-0704 and PB90-297 from the DGICYT from Spain.

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Received 31 January 1992/7 May 1992; accepted 12 May 1992