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Calcium Transport Sensitive to Ruthenium Red in Cytochrome Oxidase Vesicles Reconstituted with Mitochondrial Proteins

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Abstract

We describe a calcium transport that is sensitive to ruthenium red in liposomes reconstituted with mitochondrial extracts. This system is able to build an internally negative membrane potential, which allows the electrogenic influx of Ca^{2+} and Sr^{2+}. Proteins with molecular weights higher than 35 kDa were incorporated to the vesicles, and enhanced the accumulation of the cation in an energy-dependent fashion.

Key Words: Ca^{2+} transport; liposomes; ruthenium red; mitochondria.

Introduction

Calcium ion influx into the mitochondrial matrix appears to be mediated by a reversible uniport system, which seeks to equilibrate the electrochemical potential of Ca^{2+} across the inner mitochondrial membrane (Selwyn *et al.*, 1970; Rottenberg and Scarpa, 1974; Puskin *et al.*, 1976). This process is dependent on an internal negative $\Delta\psi$, and is inhibited by ruthenium red and lanthanides (Reed and Bygrave, 1974; Rossi *et al.*, 1973).

In the last years, several attempts have been made to purify a mitochondrial protein with clear Ca^{2+} transport activity (Sottocasa *et al.*, 1971; Gómez-Puyou *et al.*, 1972; Blondin, 1974; Fry and Green, 1979; Jeng and Shamoo, 1980; Rosier and Gunter, 1980). Sottocasa *et al.* (1971) isolated a soluble Ca^{2+}-binding glycoprotein from liver mitochondria by hypotonic shock. Antibodies prepared against this 42,000-Da protein (Panfili *et al.*,

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1979) inhibit the rate at which high concentrations of Ca^{2+} are accumulated. However, the easy extraction would indicate that it is not an integral membrane protein. In addition, the large amounts in which it may be prepared (Sottocasa *et al.*, 1971) contrast with the low number of La^{3+} binding sites reported (Reed and Bygrave, 1974). Jeng and Shamoo (1980) have isolated a 3000-Da polypeptide from mitochondria, which is highly acidic and capable of transferring Ca^{2+} into an organic phase. Another possible Ca^{2+} translocator arose as a result of experiments with reconstituted purified cytochrome oxidase (Fry and Green, 1979; Rosier and Gunter, 1980; Rosier *et al.*, 1981). Fry and Green described an increased passive Ca^{2+} permeation in liposomes reconstituted with subunit I of cytochrome oxidase, whereas Rosier and Gunter (1980) observed a slow, respiration-dependent, uptake of Ca^{2+} in vesicles reconstituted with cytochrome oxidase.

The purpose of the present work was to study the transport of Ca^{2+} in liposomes reconstituted with mitochondrial extracts. The results obtained indicate that Ca^{2+} accumulation is energy dependent and is inhibited by ruthenium red.

Materials and Methods

Preparation of Mitochondria

Mitochondria from rat kidney cortex were prepared, as described elsewhere (Chávez *et al.*, 1985), in 250 mM sucrose, 10 mM TRIS-HCl,[3] and 1 mM EDTA, pH 7.4, as isolation medium. The mitochondria were then washed in this medium without EDTA. Proteins were determined by the biuret method after solubilization of mitochondria with deoxycholate (Gornall *et al.*, 1949). Bovine serum albumin was used as standard.

Preparation of the Vesicles

Cytochrome oxidase was purified from beef heart as described (Ramírez *et al.*, 1987). COV and COV_M were obtained with the cholate dialysis method (Hinkle *et al*, 1972), using acetone-extracted asolectin (Kagawa and Racker, 1971). The dried lipids were dispersed (vortex mixing) in aqueous buffer (H_3PO_4–TEA 50 mM, pH 7.0) at a final phospholipid concentration of 30 mg/ml and sonicated to clarity. Cytochrome oxidase was then added to a

[3]Abbreviations used: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; COV, cytochrome oxidase vesicles; COV_M, cytochrome oxidase vesicles plus mitochondrial extracts; EDTA, ethylendiamine-*N*,*N*′-tetraacetic acid; $\Delta\psi$, mitochondrial membrane potential; TRIS, Tris(hydroxymethyl)aminomethane; TEA, triethanolamine; TMPD, *N*,*N*,*N*′*N*′-tetramethyl-phenylenediamine; R.R., ruthenium red; P.L., phospholipids; cit *c*, cytochrome *c*; PAGE, polyacrylamide gel electrophoresis; cyt ox, cytochrome oxidase; SDS, sodium dodecylsulfate.

final concentration of 0.25 mg/ml and mitochondrial extracts solubilized with sodium cholate to a concentration of 3 mg/ml. The incorporation of the membrane proteins was achieved after a gentle sonication for 10 sec. The suspension was dialyzed overnight at 4°C against 250 volumes of 50 mM KH_2PO_4, pH 7.0, and then passed through a G-50 Sephadex column pre-equilibrated with 50 mM KH_2PO_4, 0.8% sodium cholate, 0.5% asolectin, pH 7.0. The vesicles were collected by centrifugation at 100,000 *g* for 1.5 h and then resuspended in the dialysis buffer.

Vesicles with encapsulated dye were prepared similarly by adding Antipyrylazo III to a final concentration of 3 mM in the vortex mixing step (Kester and Sokolove, 1989).

Mitochondrial Extracts

Membrane proteins were solubilized with 1.6% sodium cholate and centrifuged in a Beckman Spinco Microfuge for 4 min at maximum speed. The supernatant, about 3 mg/ml, was loaded on the top of a glycerol step gradient (v/v) constructed with 6 ml of 50%, 6 ml of 40%, 6 ml of 30%, 6 ml of 20%, and 4 ml of 10% in 50 mM KH_2PO_4, 1 mM EDTA, 0.5 mg/ml asolectin, and 0.8% sodium cholate, pH 7.0. Mitochondrial extract (6 mg) was added and centrifuged at 90,000 *g* for 2.5 h in an SW25Ti rotor type centrifuge. Fractions of 5 ml were collected carefully using Pasteur pippetes. The fractions were incorporated to the vesicles for Ca^{2+} uptake assay. The upper fraction (10% glycerol) containing almost 20% of protein had no activity, neither did the bottom fraction (50% glycerol) which included a light pellet. The remaining volume was designated MG. Incorporated proteins were determined by the Nakamura method (Nakamura *et al.*, 1983).

Assay of Ca^{2+} Uptake

Samples were prepared in a final volume of 1 ml of 50 mM KH_2PO_4 (pH 7.0), containing 1 mg of vesicle phospholipids plus 7.5 mM ascorbate, pH 7.0, 0.75 mM TMPD, and 150 μg cytochrome *c*. To inhibit calcium uptake, purified ruthenium red was added (Luft, 1971) to a final concentration of 10 μM (unless otherwise indicated). The uptake reaction was initiated by adding 0.05 ml of 10 mM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). Using the protamine aggregation/filtration technique (Rosier *et al.*, 1979), 0.2 ml of a protamine solution (4 mg/ml) was added to precipitate the vesicles, and the sample was immediately filtered with a 0.45 μm pore size Millipore filter. This was washed immediately with 10 ml of cold 10 mM $CaCl_2$ and dried. Trapped $^{45}Ca^{2+}$ was determined using the standard liquid scintillation technique. $^{90}Sr^{2+}$ (specific activity 1000 cpm/nmol) was determined in the same way. Samples with or without ascorbate were run in parallel, and the increment or

net uptake was used to calculate the rate at the given incubation times. Ca^{2+} uptake was also determined with the encapsulated metallochromic indicator Antipyrylazo III. Measurements were performed in an SLM-Aminco DW-2000 dual-wavelength spectrophotometer at 790–720 nm (Johnson and Scarpa, 1973).

Assessment of Liposomes' Integrity

Integrity of the liposomes was determined by their ability to maintain a membrane potential; changes in $\Delta\psi$ were followed by dual-wavelength spectroscopy using safranine at 533 minus 511 nm, as reported (Akerman and Wikström, 1976).

Other Assays

Magnesium determinations were carried out in a Perkin-Elmer 560 atomic absorption spectrophotometer, by using the magnesium atomic absorption solution from Sigma as standard. Polyacrylamide gel electrophoresis of the mitochondrial extracts and of incorporated proteins in the vesicles was performed in the presence of 0.1% sodium dodecylsulfate, as described by Weber and Osborn (Weber and Osborn, 1968). Silver stain for SDS PAGE was performed as described by Oakley (1980).

Results

Early reports (Fry and Green, 1979; Rosier and Gunter, 1980; Rosier *et al.*, 1981) have shown that cytochrome oxidase vesicles, in the presence of an oxidizable substrate and cytochrome *c*, are able to accumulate Ca^{2+} in response to an internal negative membrane potential. However, this electrophoretic accumulation of Ca^{2+} is not sensitive to ruthenium red. Thus, it appears that the transport is not mediated by the uniporter. Figure 1 represents Ca^{2+} accumulation by phospholipid vesicles reconstituted with a complete mitochondrial extract and cytochrome oxidase. As observed, in COV_M vesicles, Ca^{2+} accumulation attained its maximum value, i.e., 260 nmol · mg^{-1} after 30 min of incubation time. Ruthenium red, an inhibitor of mitochondrial Ca^{2+} transport (Vasington *et al.*, 1972; Reed and Bygrave, 1974; Luthna and Olson, 1977), also inhibits calcium uptake in this reconstituted system. When ruthenium red was added, the uptake value decreased to 190 nmol mg^{-1}. This result seems to indicate that calcium transport in COV_M was carried out through the Ca^{2+} uniport system. This statement is reinforced by the fact that energized vesicles without mitochondrial proteins failed to accumulate high amounts of the cation. The increment in Ca^{2+}

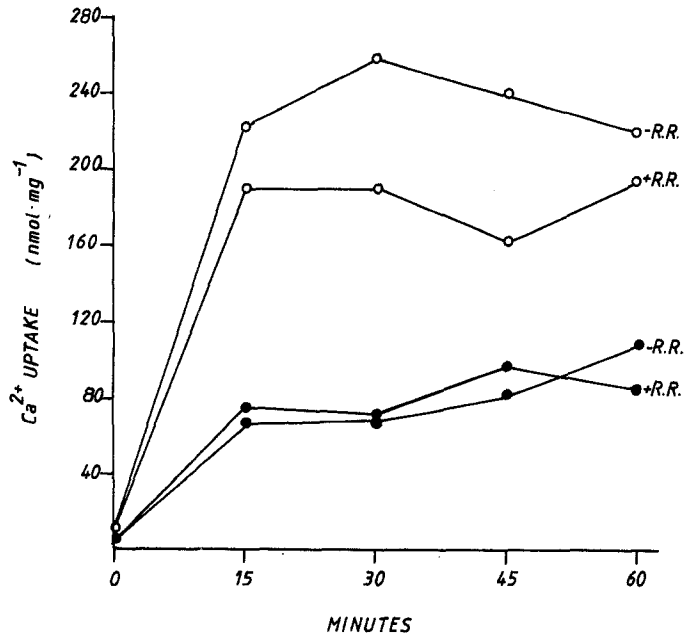

Fig. 1. Ca^{2+} uptake in COV and COV_M as a function of time. A 1-mg portion of cytochrome oxidase vesicles was added to an incubation medium containing 50 mM KH_2PO_4, 7.5 mM ascorbate, 0.75 mM TMPD, and 150 μg cytochrome *c* (pH 7.0). Where indicated, 4 μM ruthenium red was added. Samples were incubated during the indicated time and then filtered through 0.45 μm Millipore filters using the protamine aggregation/filtration technique. Filled circles (●) represent cytochrome oxidase vesicles and open circles (○) represent cytochrome oxidase vesicles with incorporated mitochondrial proteins from a complete membranous extract (5 mg/ml) solubilized in 1.6% sodium cholate, before dialysis. Final volume 1 ml; temperature 25°C.

transport in COV_M was twice that observed in COV. The effect of ruthenium red in COV was negligible, which agrees with the findings of Rosier and Gunter (1980). Figure 2 summarizes the inhibition of calcium uptake in COV_M by R.R. This plot is linear with an apparent dissociation constant for the inhibitor (K_i) of 3.5 μM at pH 7.0. Additionally, Ca^{2+} uptake at short times was explored; accumulation was 120 nmol Ca^{2+}/mg during the first minute of incubation. Qualitative measurements of membrane potential in COV and COV_M were made using the membrane potential dye, safranine, according to methods previously reported (Akerman and Wikström, 1976). Figure 3a shows that the membrane potential was achieved only when the vesicles were energized by oxidation of ascorbate. Interestingly, Ca^{2+} addition abolished the internal negative membrane potential developed in

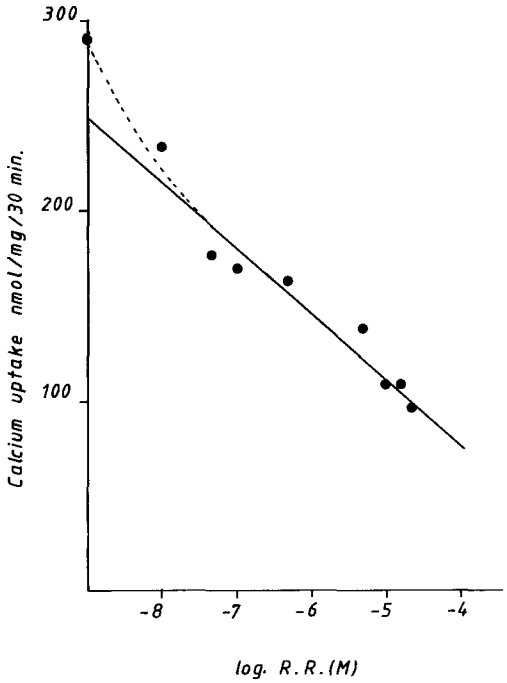

Fig. 2. Inhibition of calcium transport by ruthenium red in cytochrome oxidase vesicles with incorporated mitochondrial proteins. Samples were assayed as in Fig. 1. Final volume 1 ml; temperature 25°C.

COV. Therefore, Ca^{2+} transport seems to occur through an electrogenic mechanism.

The fact that ruthenium red diminished the effect of Ca^{2+} on $\Delta\psi$ (Fig. 3b) reinforces the statement that the Ca^{2+} translocator was incorporated in COV_M. The deflection, following ruthenium red addition, corresponds to absorbance of the dye at 533–511 nm. Quantitative Ca^{2+} uptake in reconstituted systems is shown in Table I. The net uptake of Ca^{2+} in vesicles with incorporated mitochondrial extracts was around 270 nmol Ca^{2+}/mg of protein/30 min, supported by internally negative $\Delta\psi$ in vesicles of cytochrome oxidase. These values were reduced 50% when ruthenium red was present in the assay. It should be noted that in each experiment all net uptake rates and ruthenium red-insensitive uptake were calculated as the difference between the energized and nonenergized uptake rates. The uptake rates in COV were also subtracted in each situation. The average uptake rate in energized COV was 2.42 $\pm$ 0.9 nmol Ca^{2+}/mg of cyt ox/min. This value is

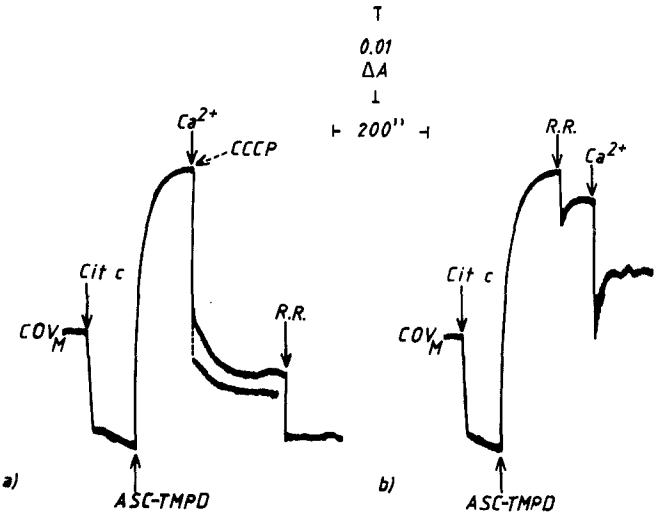

Fig. 3. Collapse of $\Delta\psi$ as induced by Ca^{2+} or CCCP in cytochrome oxidase vesicles. COV_M represents cytochrome oxidase vesicles with incorporated mitochondrial proteins from a complete membranous extract, solubilized in 1.6% sodium cholate, as described in Materials and Methods, before dialysis. A 1-mg portion of COV_M was suspended in 50 mM KH_2PO_4, and 10 μM safranine was used as indicator. Where indicated, 150 μg cytochrome *c*, 7.5 mM ascorbate, 0.75 mM TMPD, 100 μm $CaCl_2$, 10 μM CCCP, or 10 μM ruthenium red (R.R.) were added. Final volume 1 ml; temperature 25°C.

Table I. Inhibition by Ruthenium Red of the Reconstituted Ca^{2+} Uptake[a]

Incorporated fraction into COV	nmol $^{45}Ca^{2+}$/mg protein/30 min		
	Net uptake (−R.R.)	R.R. insensitive (+R.R.)	R.R. sensitive
COV_M	283.6 ± 50.4 (7)	132.2 ± 66.0 (7)	151.4 (53.3%)
COV_{MI}	285.0 ± 153.0 (3)	139.8 ± 96.0 (3)	144.9 (50.9%)
COV_{MG}	201.0 ± 15.0 (3)	93.9 ± 27.0 (3)	108.3 (53.6%)

[a]Cytochrome oxidase vesicles were prepared, as previously described, with the indicated proteins in the suspension at the time of dialysis. The ruthenium red-sensitive uptake rate is the difference between the net uptake and the ruthenium red-sensitive uptake. COV_M represents cytochrome oxidase vesicles with incorporated proteins from a complete extract of membrane proteins in 1.6% sodium cholate; COV_{MI} is the fraction of mitochondrial proteins solubilized in 1.2% detergent reconstituted in cytochrome oxidase vesicles, and COV_{MG} indicates vesicles with an extract obtained after glycerol gradient fractionation. Values in the first two rows represent the mean of the experiments indicated by the number in parenthesis ± S.D. The third row represents the calcium transport, sensitive to ruthenium red, as the difference between net uptake and calcium transport insensitive to ruthenium red.

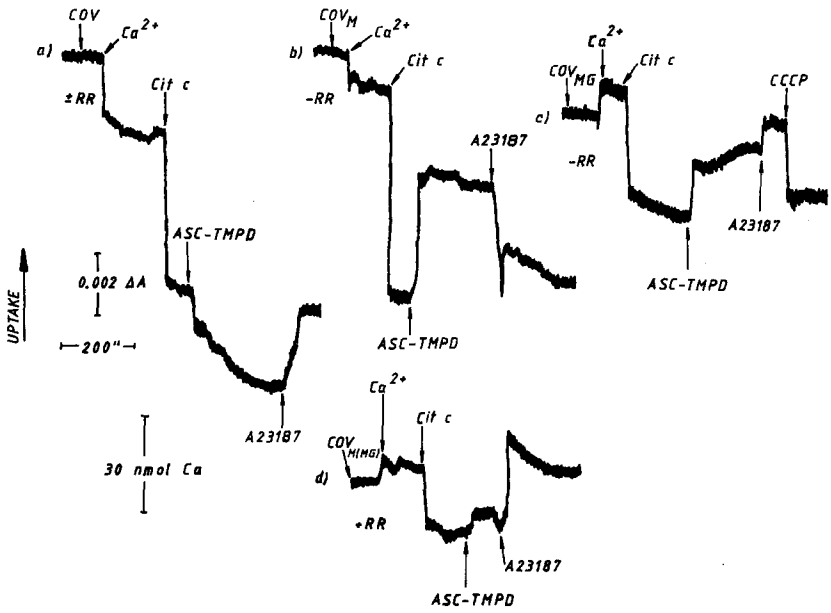

Fig. 4. Reconstitution of Ca^{2+} uptake in cytochrome oxidase vesicles with extracts of mitochondrial proteins, loaded with Antipyrylazo III. A 1-mg portion of cytochrome oxidase vesicles with incorporated proteins was suspended in 50 mM KH_2PO_4. Where indicated, 750 μM $CaCl_2$, 150 μg cytochrome *c*, 7.5 mM ascorbate, 0.75 mM TMPD, and 10 μM CCCP were added. Traces a, c, and e represent incubation in the presence of 10 μM ruthenium red.

similar to that obtained by Rosier *et al.* (1981), i.e., 2.9 nmol Ca^{2+}/mg of cyt ox/min in internally negative $\Delta\psi$ COV.

To determine whether the data in Table I corresponded to Ca^{2+} transport or represented Ca^{2+} binding, calcium uptake was followed by using encapsulated Antipyrylazo III in the incorporated systems (Fig. 4). Antipyrylazo III was chosen, instead of the more classic metallochromic indicator Arsenazo III (Kester and Sokolove, 1989), because it allows measurement of Ca^{2+} transport even in systems with colored reagents (cit c). As observed (Fig. 3a), in energized vesicles without mitochondrial extracts no increments in absorbance of the entrapped indicator, Antipyrylazo III, occurred. The latter indicates that calcium was not accumulated in these vesicles. The neutral ionophore, A23187, promotes Ca^{2+} uptake despite incubation with ruthenium red. Addition of ascorbate-TMPD to the vesicles, with incorporated mitochondrial extracts (COV_M and COV_{MG}), induced an important increment in Ca^{2+} uptake. This was more striking in COV_M (Fig. 4, trace b), since these vesicles incorporated approximately 40 μg of protein/mg of P.L. from the complete mitochondrial extract (M). Conversely, COV_{MG} only

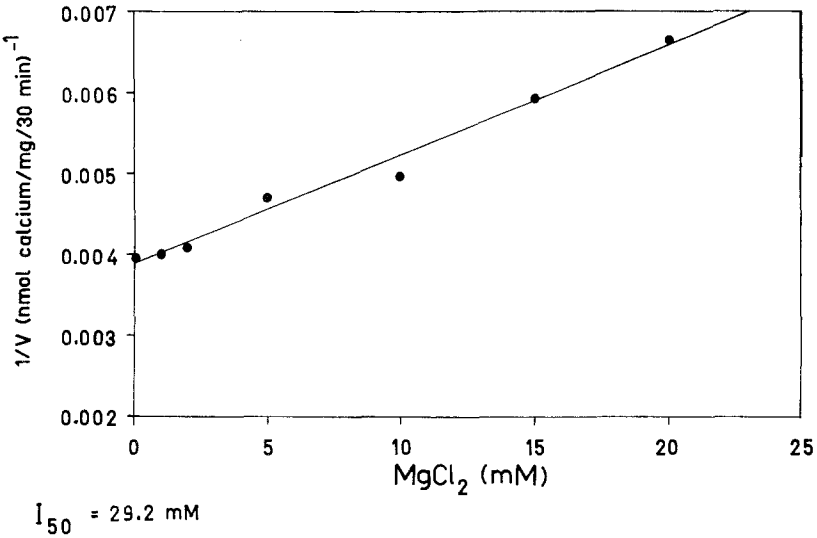

Fig. 5. Inhibitory effect of magnesium on calcium uptake in cytochrome oxidase vesicles with incorporated mitochondrial extracts. Calcium transport was assayed as described in Materials and Methods. Final volume 1 ml; temperature 25°C.

incorporated around 20 μg/mg of P.L. (Fig. 4, trace c). When ruthenium red was present in the medium, a clear inhibition in Ca^{2+} uptake occurred in both samples (Fig. 4d).

It is known from the work of Scarpa and Azzone (1968) that, in liver mitochondria, the surface binding of calcium is reduced by the addition of Mg^{2+}, by lowering pH, or by increasing the osmolarity of the medium. Figure 5 shows the effect of Mg^{2+} in calcium uptake. A 50% inhibition (I_{50}) at 29.2 mM was calculated from a Dixon plot. The inhibition at this concentration suggests that magnesium binds at the level of the membrane surface, producing screening effects as occurs in rat liver mitochondria (Vaino *et al.*, 1970). Table II compares the transport of divalent cations by the reconstituted system. Calcium and strontium were transported in an energy-dependent fashion and their accumulation was ruthenium red sensitive, i.e., $^{90}Sr^{2+}$ uptake was inhibited by ruthenium red in 34.7%. Again, the uptake rates were calculated as the difference between the energized and nonenergized uptake rates. Magnesium influx into vesicles was determined by atomic absorption, but no differences in the energized and nonenergized samples were found. Figure 6 shows the scanning of the mitochondrial extracts used in the incorporation, as resolved in SDS gel electrophoresis. In the fraction from the extract obtained by fractionation with glycerol gradient

Table II. Divalent Cation Transport in COV_M[a]

Divalent cation transport in COV_M	nmol/mg protein/30 min	
	−R.R.	+R.R.
Ca^{2+}	283.6 ± 50.4 (7)	132.1 ± 66.0 (7)
Sr^{2+}	239.9 ± 92.3 (3)	156.7 ± 58.6 (3)
Mg^{2+}	–	–

[a]Cation transport was determined as described in Materials and Methods. The uptake rates are the difference between the uptake in energized and nonenergized vesicles. Final volume 1 ml; temperature 25°C.

(MG), a significant amount of low-molecular-weight proteins was eliminated. Finally, Fig. 7 shows the polyacrylamide gel electrophoresis of the cytochrome oxidase vesicles, reconstituted with mitochondrial extracts, compared with molecular weight standards (well a). Well b shows total mitochondrial proteins. Well c illustrates the proteins reconstituted from the complete extract, and well e shows those incorporated in the vesicles from the glycerol extract. Well d shows a glycerol gradient extract that has no activity. Proteins with molecular weights higher than 35 kDa were preferently stained in samples c and e. Simultaneously, vesicles with incorporated cytochrome oxidase were electrophoresed. The amount of protein incorporated could not be observed using Coumassie Blue staining (not shown).

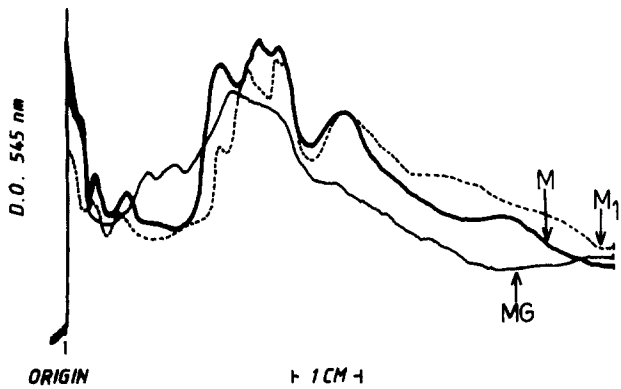

Fig. 6. Scanning of mitochondrial extracts before incorporation into COV. Rat kidney mitochondria were solubilized with sodium cholate: M represents a complete extract of membranous protein in 1.6% detergent; M1 is the fraction soluble in 1.2% sodium cholate, and MG is an extract obtained after glycerol gradient separation; 150 μg of each sample were electrophoresed, as described in Materials and Methods, and stained with 0.1% Coomassie Blue.

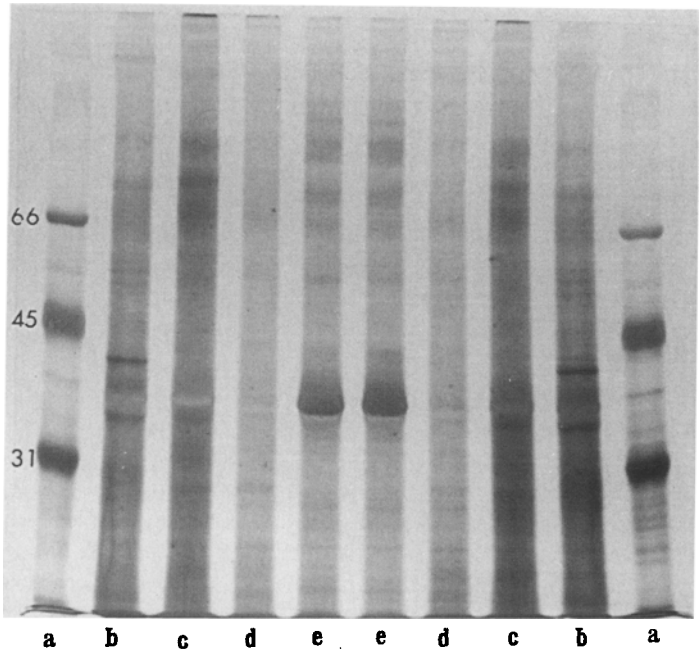

Fig. 7. SDS PAGE (10% gel) of cytochrome oxidase vesicles reconstituted with mitochondrial extracts. Samples of 15 μg protein were incubated with 0.1% SDS and 0.1% β-mercaptoethanol in a 100°C water bath for 2 min. Then the solution was mixed with 10 μl Bromophenol Blue (2.5%) and placed into the wells. Molecular weight standards (well a) and a complete mitochondrial extract (well b) were also electrophoresed as controls.

Discussion

Several authors have demonstrated Ca^{2+} uptake into cytochrome oxidase vesicles reconstituted by using phospholipids and extracted mitochondrial proteins (Gunter *et al.*, 1978; Fry and Green, 1979; Rosier and Gunter, 1980; Rosier *et al.*, 1981; Saltzgaber-Müller *et al.*, 1980). Rosier and Gunter (1980) observed an energy-dependent uptake of Ca^{2+} in cytochrome oxidase vesicles. Ruthenium red does not appreciably affect calcium uptake in such vesicles. However, agents that break down the proton gradient developed in COV, such as CCCP or the combination of nigericin, valinomycin, and K^+ (Fry and Green, 1979), were found to be potent inhibitors of Ca^{2+} accumulation. The lack of effect of ruthenium red on the Ca^{2+} uptake in COV may indicate, as proposed by Rosier and Gunter (1980), that either modification of the calcium mediator has occurred or that Ca^{2+} transport in COV is mediated by an entirely different, perhaps less specific, transport system. In

1981, Rosier *et al.* demonstrated uptake of calcium against its electrochemical gradient into internally positive asolectin vesicles containing cytochrome oxidase and fraction V, which includes the high-affinity uncoupler site as well as the base protein (F_0) of the mitochondrial H^+-transporting ATPase (F_1). Calciphorin, a putative calcium ionophore protein of 3000 Da molecular weight (Jeng and Shamoo, 1980), was tentatively identified as the electrophoretic uniporter involved in Ca^{2+} uptake in mitochondria, but Sokolove and Brenza (1983) found that all of the Ca^{2+} binding properties of the crude hepatic calciphorin fraction can be attributed to the associated lipids. Cardiolipin is a major component in mitochondrial membranes. Tyson *et al.* (1976) demonstrated that cardiolipin, when present in an organic phase at millimolar concentrations, can mediate the transport of calcium in a Pressman cell. In our assays, the lipid constituents probably cannot account for the transport rates that we observed, since we reconstituted both lipid and protein components in each sample. As indicated, we report the energy-dependent calcium transport, not the binding of calcium to the whole system. A chymotrypsin-sensitive polypeptide of 8800 Da, an impurity in cytochrome oxidase preparations (Saltzgaber-Müller *et al.*, 1980), was also incorporated in COV; however, ruthenium red, at concentrations of 100 μM, did not inhibit Ca^{2+} uptake. The present work shows that high-molecular-weight proteins (35–60 kDa) from rat kidney mitochondrial extracts are preferentially reconstituted in COV. These proteins enhance Ca^{2+} accumulation as well as Sr^{2+} uptake in cythocrome oxidase vesicles in an energy-dependent fashion. Calcium accumulation seems to be related with the so-called uniporter since it is sensitive to ruthenium red; although the dye levels could seem too high, most of them could be acting on unspecific binding to phospholipids. In agreement with our results, Zimniak and Barnes (1980) have reported the characterization of an electrogenic calcium transporter in membrane vesicles from *Azotobacter vinelandii*, which is inhibited by 4 μM of ruthenium red at pH 6.5.

The inhibition of calcium transport by high concentrations of Mg^{2+} could indicate binding or charge screening effects. On the basis of inhibitor studies (Crompton *et al.*, 1976), influx of magnesium into heart mitochondria is believed to occur via a mechanism distinct from the Ca^{2+} uptake mechanism (Diwan, 1987), whereas calcium transport inhibitors inhibit Mg^{2+} as well as Ca^{2+} influx in brain and in liver mitochondria (Rugola and Zoccarato, 1984; Kun, 1976). Thus, although the calcium accumulation system has a very low activity for Mg^{2+} (Vaino, *et al.*, 1970), complete exclusion of this cation from transport on the uniporter may be tissue specific.

The reconstituted system presented in this work shows cation selectivity, since Ca^{2+} and Sr^{2+} were transported, whereas Mg^{2+} was not (Table II). These results are in agreement with the selectivity series of the uniporter,

known as $Ca^{2+} > Sr^{2} > Mn^{2+} > Ba^{2+} > La^{3+} \gg Mg^{2+}$. This series is not followed by the system proposed by Jeng and Shamoo (1980), since Mn^{2+} and Zn^{2+} interact strongly with calciphorin, whereas Sr^{2+} and Mg^{2+} show a slight effect on calcium extraction by calciphorin (Sokolove and Brenza, 1983).

We cannot discount the fact that some of the possible Ca^{2+} translocators, already cited, were reconstituted in this system. What is true is that the characteristics of those systems alone cannot account for the current data. The question remains if a single band (35 kDa) stained from the incorporated proteins of the pooled extracts could correspond to the glycoprotein isolated by Sottocasa *et al.* (1971). The molecular nature of the calcium transporter is unknown. By analogy with other systems, it is most likely a protein, but at present we cannot exclude other possibilities, e.g., a low-molecular-weight ionophore or lipid molecules (Tyson *et al.*, 1976).

Considerable effort has been made to elucidate the nature of the uniporter (Blondin, 1974; Fry and Green, 1979; Jeng and Shamoo, 1980; Rosier *et al.*, 1981; Reed and Bygrave, 1974; Sottocasa *et al.*, 1971; Sandri *et al.*, 1976; Panfili and Crompton, 1983; Saltzgaber-Müller *et al.*, 1980), but there is still no clear evidence for the isolation and reconstitution of the Ca^{2+} mitochondrial uniporter.

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