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Advances in the Purification of the Mitochondrial Ca^{2+} Uniporter Using the Labeled Inhibitor $^{103}Ru_{360}$[1]

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For many years the calcium uniporter has eluded attempts of purification, partly because of the difficulties inherent in the purification of low-abundance hydrophobic proteins (Reed and Bygrave, 1974). Liquid-phase preparative isoelectric focusing improved the fractionation of mitochondrial membrane proteins. A single 6-h run resulted in a 90-fold increase in specific activity of pooled active fractions over a semipurified fraction, allowing for enrichment of the calcium transport function in cytochrome oxidase vesicles. An additional powerful tool in the isolation of the uniporter was the use of the labeled inhibitor $^{103}Ru_{360}$ as an affinity ligand; by following this procedure a protein of 18 kDa was purified in nondenatured, but rather inactive, form. The labeled protein corresponds to the protein that showed Ca^{2+} transport activity.

KEY WORDS: Mitochondria; Ca^{2+} uniporter; calcium transport inhibitors.

INTRODUCTION

The currently proposed roles for intramitochondrial calcium transport (Hansford, 1988; McCormack and Denton 1989) emphasize the importance of Ca^{2+} accumulation and, thereby, a renewed interest in the investigation of the uptake mechanism. The uptake carrier is an uniporter that reduces extramitochondrial calcium concentration within the concentration of 10^{-7} to 10^{-5} M; the rate of uptake is a function of calcium concentration showing sigmoidal kinetics, a $K_{0.5}$ which depends on the membrane potential and a Hill coefficient near 2.0 (Gunter and Pfeiffer, 1990). This system is inhibited by low concentrations of lanthanides and ruthenium red (Reed and Bygrave, 1974). Recently, Ying *et al.* (1991), reported the synthesis of an oxo-bridged ruthenium complex that possesses higher inhibitory activity than ruthenium red itself.

In contrast with the remarkable progress in the field of metabolic regulation by calcium, attempts to identify the components of the calcium transport system with the ultimate goal of isolating them and reconstituting the process, still remains to be done. The low uniporter concentration, estimated between 0.001–0.01 nmol/mg protein, constitutes a serious problem for its isolation, as well as the selection of the adequate detergent to remove the uniporter from the lipidic environment without denaturing the protein. Even with these difficulties, however, there has been some success in isolating, identifying, and reconstituting this porter (Gunter and Pfeiffer, 1990). Saris *et al.* (1993) found that antibodies raised against a glycoprotein–peptide complex inhibited the calcium uniporter in rat liver mitoplasts. Mironova *et al.* (1994) reported the purification of the channel-forming component of that complex and described its channel properties when reconstituted into a planar lipid bilayer. Our laboratory reported the identification of a low molecular-weight protein, i.e., 20 kDa, by using specific antibodies that inhibit calcium transport by 60% in rat liver mitoplasts (Zazueta *et al.*, 1994). In this paper we present results

[1] Abbrevations used: TEA, Triethanolamine; CHAPS, (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate); TMPD, tetramethyl-l-phenylendiamine; COV, cytochrome oxidase vesicles; SMP, submitochondrial particles; $C_{12}E_8$, mono-*N*-dodecyloctaetilenglycol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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of purifying the mitochondrial calcium uniporter using a specific ligand: the high-affinity inhibitor $^{103}Ru_{360}$ synthesized from a radioactive precursor, as well as experiments of reconstitution in cytochrome oxidase vesicles.

MATERIAL AND METHODS

Rat kidney mitochondria from adult female Wistar rats were obtained as described (Chávez *et al.*, 1985). Submitochondrial particles were obtained by the method of Lee and Ernster (1965).

Solubilization of Rat Kidney Submitochondrial Particles

SMP (3 mg/ml) were solubilized in 1.2% sodium cholate (w/v) in a sucrose medium. Solubilization was carried out with constant stirring for 30 min at 4°C. The material was centrifuged for 1 h at 100,000 × *g*. The supernatant was collected for further fractionation and reconstitution.

Fractionation of Submitochondrial Particles

Solubilized SMP (3 mg/ml) were saturated with 50% ammonium sulfate and centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was then 90% saturated with ammonium sulfate and centrifuged at 20,000 × g for 15 min at 4°C. The resultant pellet was homogenized with 50 mM KH_2PO_4, pH 7.0, and dialyzed against the same buffer in a 1:250 proportion (F_{90} fraction).

Polyclonal antibodies were obtained against this fraction (Zazueta *et al.*, 1994) and the purified IgGs were immobilized in an Affi-Gel hydrazide column. This affinity column was used to enrich the F_{90} fraction by successive passings of solubilized submitochondrial particles in 1.0% CHAPS and eluting the recognized proteins with 150 mM sodium citrate, pH 5.0 (Fig. 1).

Liquid-Phase Isoelectric Focusing

The enriched preparation (8 mg, 30 ml) was desalted using the ion retardation ECONOPAC 10DG desalting column and diluted to a final volume of 55 ml containing 0.5% CHAPS (w/v), 20% glycerol, and 2 ml of BioLyte ampholites, pH range 5–8. This solution was loaded into a liquid–phase isoelectrofocusing Rotofor Cell without further treatment. Focusing was carried out at 12-W constant power for 6 to 7 h at 4°C. At equilibrium, the values were 740 V and 16 mA. Twenty fractions were harvested and their pH values measured. Protein concentration was determined by a modified Lowry procedure (Nakamura *et al.*, 1983).

Reconstitution of Liquid-Phase Isoelectrofocused Proteins

After measurement of pH and protein concentration, samples were incubated with 1 M NaCl (final concentration) and dialyzed against 250 volumes of 50 mM KH_2PO_4, pH 7.0, to eliminate ampholites. Each fraction (60–90 μg) was incubated with 0.5 ml of COV), as described below, and sonicated in a water sonicator for 3 to 5 s in the presence of 0.5% sodium cholate. The detergent was removed by dialysis or by dilution with 40 volumes of 50 mM KH_2PO_4, pH 7.0, followed by ultracentrifugation at 45,000 rpm for 1 h. The liposomes were resuspended in the same buffer and the activity of reconstituted calcium uptake was analyzed.

Calcium Uptake Activity in Cytochrome Oxidase Vesicles

Dried lipids (40 mg asolectin) were sonicated to clarity in 50 mM H_3PO_4–TEA, pH 7.0. Cytochrome oxidase was added to a final concentration of 0.25 mg/ml and incorporated by simple mixing with the liposomes, as described by Ramírez *et al.* (1987). COV (40 mg/ml), with incorporated proteins from mitochondrial extracts, were added to an incubation medium containing 50 mM KH_2PO_4, 7.5 mM ascorbate, 0.75 mM TMPD, 150 μg cytochrome *c* (pH 7.0), and 0.5 mM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). The samples were incubated during 5 min and an aliquot filtered through a 0.45-μm Millipore filter using the protamine filtration technique (Rosier *et al.*, 1979). Specific activity values represent the difference between energized (plus cytochrome oxidase substrates) and nonenergized uptake rates.

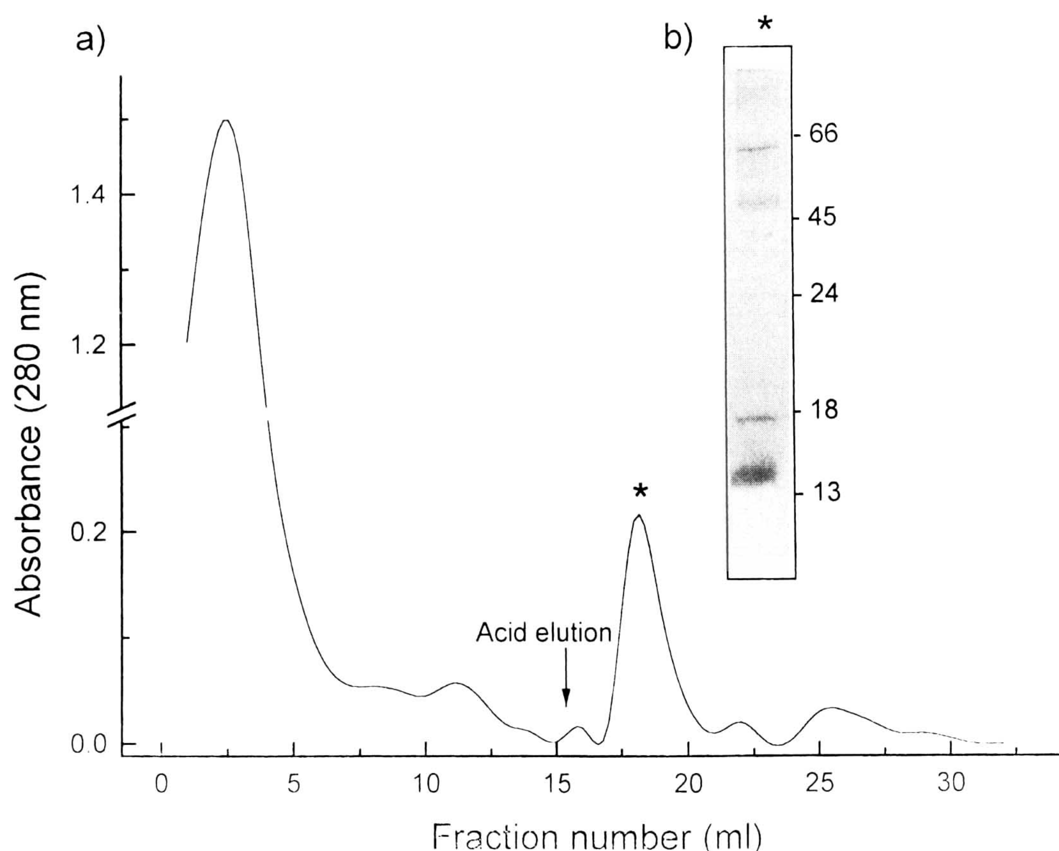

Fig. 1. Enrichment of the F_{90} fraction by immunoaffinity chromatography. (a) Elution profile of SMP after passing them through the immunoaffinity column. Polyclonal antibodies were raised against the fraction precipitated with ammonium sulfate to 90% saturation. These antibodies were coupled to a Affi-Gel hydrazide column and equilibrated with 10 m*M* Tris, 0.1% CHAPS, pH 7.5. Semicrude extracts (1 or 2 mg) solubilized in the same buffer were used. The proteins that freely passed through the column were eliminated and the recognized ones were eluted with 150 mM sodium citrate, 0.3% CHAPS, pH 5.0. (b) Silver staining of the eluted fraction. Of this fraction, 10 μg were electrophoresed in a 15% polyacrylamide gel in denaturing conditions and stained as described in the methods section.

Synthesis of the Radiolabeled Ru_{360}

The synthesis of the inhibitory complex was based on the method for the preparation of ruthenium red, as modified by Ying *et al.* (1991). The radiolabeled Ru_{360} was synthesized from 2.5 mg $RuCl_3 \cdot 3\ H_2O$ combined with 1 mCi of $^{103}RuCl_3$. The final separation was achieved using a 0.5 × 3.0 cm column packed with carboxymethyl cellulose. The yield was 130 nmol of Ru_{360} as calculated from the molar coefficient extinction of the complex at 360 nm, i.e., 2.6×10^4 M^{-1} cm^{-1}.

Inhibition of Mitochondrial Calcium Uptake

Calcium uptake was followed by incubating mitochondria with $^{45}CaCl_2$ (specific activity 1000 cpm/nmol) in the presence of increasing concentrations of Ru_{360}. After 1 min, an aliquot of the sample was filtered and its radioactivity measured in a scintillation counter. The assay was carried out under three substrate concentrations. Calculations of the lines in the Dixon plots and line fittings were carried out with the Microcal Origin computer program (Microsoft Corp.). The linear-regression coefficient of calculated lines varied between 0.95 and 0.99.

Binding of $^{103}Ru_{360}$ Complex to Intact Mitochondria

Mitochondria were incubated in 6 ml of 250 mM sucrose/10 mM HEPES/10 mM succinate, pH 7.3, at different concentrations of $^{103}Ru_{360}$. After 10 min, the

samples were centrifuged for 10 min at 13,000 rpm and the pellets were washed with 10 ml of medium to eliminate free inhibitor. To remove nonspecific binding, 1.5 μM unlabeled inhibitor was added to mitochondria; after 10 min, the samples were centrifuged and washed as above, for radioactivity measurement.

Affinity Chromatography of the Membrane Proteins

Submitochondrial particles were labeled with 10 pmol/mg $^{103}Ru_{360}$ in the presence of 1.0% $C_{12}E_8$ or 1% CHAPS for 30 min at 0°C. The labeled proteins were separated by CM-cellulose chromatography, using a linear gradient of ammonium formate, pH 5.5.

SDS–PAGE Analysis of Protein Fractions

Polyacrylamide gel electrophoresis was performed in the presence of 2% sodium dodecyl sulfate, as described by Laemmli (1979). Other techniques include the separation in polyacrylamide in nondenaturing conditions, using the anodic discontinuous buffer system described by Davis (1964).

RESULTS

Liquid-Phase Preparative Isoelectrofocusing Separation

Rat kidney mitochondria was solubilized and treated as described in the section on material and methods, before isoelectrofocusing was performed.

Two assays methods were used for localizing the transporter following purification in the isoelectrofocusing chamber. One of them was the traditional method of solubilization and functional reconstitution into liposomes. The other one was by the Western blot technique using antibodies raised against the fraction with maximal Ca^{2+} transporting activity. The antibodies were also used to inhibit cation uptake in mitoplasts.

Figure 2a shows the pH profile and protein distribution after reaching the equilibrium in the fractionating cell. The bulk of protein was focused at pHs above 8.0; in these fractions the main protein was cytochrome *c*, as was demonstrated by analyzing its absorption spectrum and by its stained image (mol. wt. 13,000 Da) in SDS–PAGE (not shown). Interestingly, this

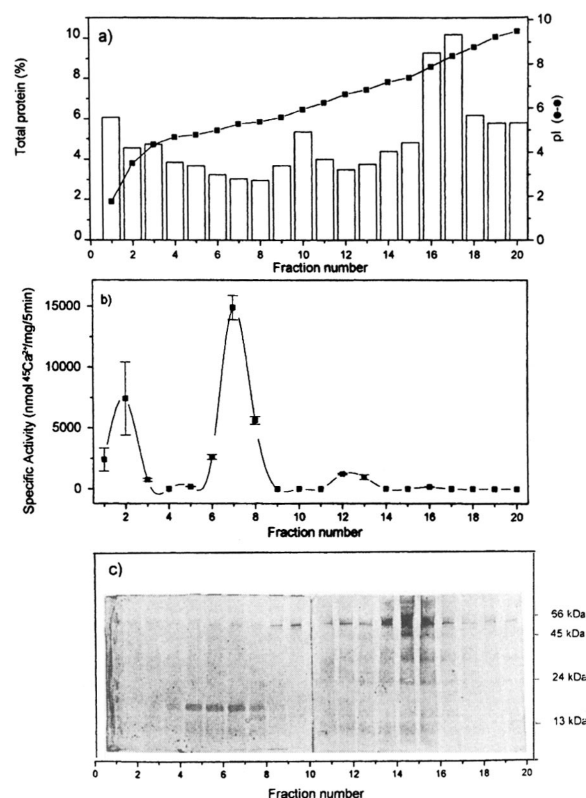

Fig. 2. (a) Analysis of isoelectrofocusing fractions by pH and protein assay. Enriched fraction (6 mg) was focused in the presence of 0.6% glycerol and 0.1% CHAPS. pH was measured immediately after collecting fractions and protein assayed by a modified Lowry technique. The ratio volts/hour (V/H = 4500) was maintained in all the focusing experiments. (b) Specific activity determination and (c) silver staining imaging of the isoelectrofocused fractions. Each of the 20 fractions obtained were assayed for calcium uptake activity as described in the methods section. Data represent mean values of three experiments ± S.D. After concentration and dialysis, 5 μg of each fraction were electrophoresed in a 15% polyacrylamide gel (2% SDS) and stained with the silver technique already mentioned.

peripheral protein was not solubilized in the primary steps of purification, which was carried out by high-salt concentration precipitation.

Each fraction was concentrated and incorporated into COVs as described earlier. After reconstitution, transport activity was measured as the ability of COVs to support uptake of $^{45}Ca^{2+}$ in presence of cytochrome *c* and the electron donor pair ascorbate–TMPD (in each case, COVs without these substrates were used as negative controls to account for passive $^{45}Ca^{2+}$ uptake and nonspecific binding). The reaction was stopped by addition of protamine sulfate, which promotes aggregation of the liposomes. An aliquot is fil-

tered and its radioactivity measured. The fractions that promoted maximal accumulation were those with isoelectric points between 5.0–6.0 (Fig. 2b). The electrophoretic analysis of the fractions with Ca^{2+} transporting activity showed an approximately 20 and a 70 kDa protein (Fig. 2c). It appears that the activity of these proteins is critically maintained above pH 5.0–6.0. The drop to the acidic end of the gradient inactivates these proteins (as observed in the activity analysis), in spite of the mild conditions used during the reconstitution and calcium accumulation assays.

A nondenaturing electrophoresis was done to determine if the proteins observed in the presence of sodium dodecyl sulfate were independent proteins or were subunits that belong to a multimer. Figure 3 shows the silver staining imaging of the ten acidic fractions. As observed from fraction 5–8, only a broad band is detected and no aggregates were formed in the front of the gel.

Inhibition by Antibodies of Calcium Uptake in Mitoplasts

To strengthen the evidence of the participation of these proteins in the calcium uptake mechanism, purification of substantial amounts of these fractions were carried out until it was possible to inject enough antigen in rabbits to raise high serum titers. As the yield of protein was poor each time (200–220 μg), the induction of antibody response was stimulated by injecting the antigen in the popliteal ganglion of female New Zealand rabbits.

The lymphocytes were confronted with the antigen in the lymph node, as the site of greatest concentration, so the primary response was greater than the one obtained when the lymphocytes left the recirculating pool and migrated to the site of antigen concentration.

Titers were determined by an indirect ELISA method (not shown). The titers were defined as the highest dilution at which the ELISA response was twice the blank value of the test. A maximal titer of 1:3200 was obtained, confronting the absorbance values (490–630 nm) obtained by the incubation of preimmune and hyperimmune serum with semicrude antigens.

The ability of antibodies to inhibit Ca^{2+} uptake in mitoplasts is illustrated in Fig. 4. Trace A shows Ca^{2+} accumulation in control mitochondria, depending on the membrane potential; it is shown that the uncoupler CCCP, by dissipating the proton gradient, releases the cation to the medium. The preimmune serum does not affect the ability of mitochondria to transport cal-

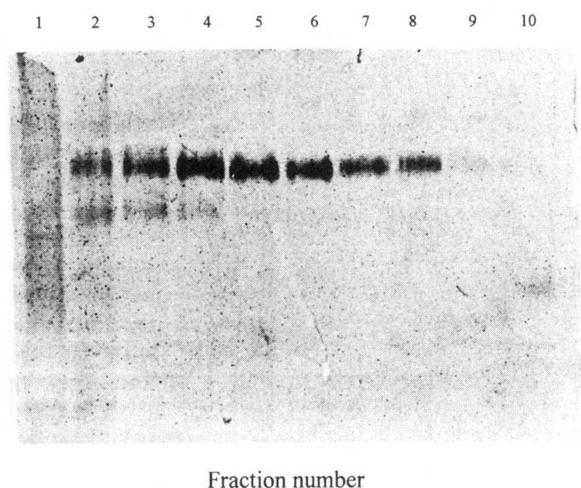

Fig. 3. Acidic fractions (10 μg) were run, at constant 200 V, in a acidic discontinuous buffer system in a 7.5% gel without SDS. To ensure the entrance of hydrophobic proteins, 0.6% CHAPS was added. Silver staining was used to detect proteins.

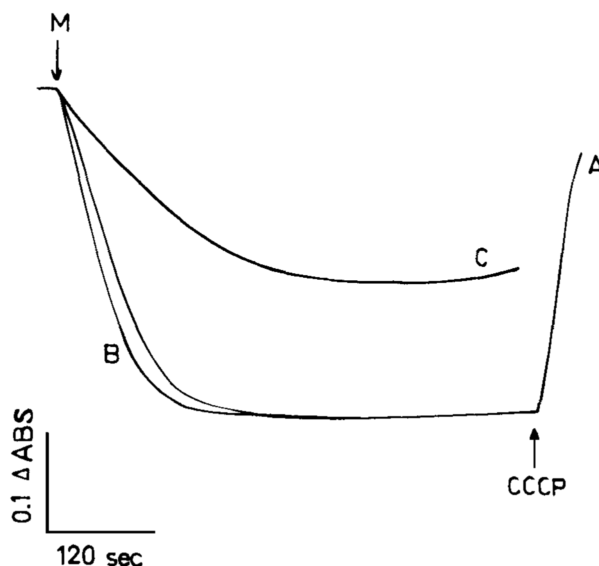

Fig. 4. Inhibition of calcium uptake by hyperimmune serum in mitoplasts. Protein (1 mg) was added to an incubation medium containing 250 mM sucrose, 10 mM succinate, 10 mM HEPES, 50 μM $CaCl_2$, 200 μM ADP, 10 μg rotenone, 2 mM phosphate, 50 μM arsenazo III, and incubated in presence of hyperimmune or control serum. Double-wavelength spectrophotometric tracings were obtained at 685–875 nm. Trace A represents calcium accumulation without serum addition; trace B was in the presence of preimmune serum and trace C was in presence of hyperimmune serum. Final volume 3 ml; temperature 25°C; pH 7.3.

cium (Fig. 4, trace B); however, in remarkable contrast, the hyperimmune serum inhibits such a reaction at the same concentration (Fig. 4, trace C). It should be mentioned that there was no inhibition of the respiratory rate of mitoplasts preparations in either condition (not shown). Control mitoplasts transported 52.80 ± 15.29 nmol Ca^{2+}/mg protein/5 min. A slight inhibition was observed when preimmune serum was incubated with mitoplasts for 1 min. The hyperimmune serum reduced calcium accumulation by 70%, i.e., to 15.93 ± 2.48 nmol Ca^{2+}/mg protein/5 min.

As expected, the analysis by immunoblotting using submitochondrial particles from rat kidney, showed two proteins of approximately 20 and 70 kDa; there is no evidence of the presence of these proteins in the membranes incubated with the preimmune serum. The hyperimmune serum recognized the same proteins when the antigen electrophoresed and blotted, as obtained from beef kidney submitochondrial particles (not shown).

The enrichment of the calcium uniporter activity in COVs is documented in Table I. Preextraction with ammonium sulfate precipitation in presence of sodium cholate, followed by isoelectrofocusing in liquid phase with a zwitterionic detergent (CHAPS), led to a substantial purification of this protein. The purification factor was 90 and the yield was 0.01%. Considering that the number of uniporters in the membrane has been estimated by inhibitor titration to be approximately 0.001 nmol/mg protein, the purification efficiency reported here is adequate. Very likely, the purification factors may be even higher, due to partial inactivation of the transport function during the procedure. On the other hand, inhibitor-binding components could be inaccessible to ruthenium red in the reconstituted system. This latter effect can be assumed by the fact that in this model the classical suppressor of calcium uptake in mitochondria inhibited calcium accumulation at concentrations one order of magnitude higher than those required to inhibit the process in the organelle (Table I). Elsewhere we reported an apparent dissociation constant for the inhibitor (K_i) of 3.5 μM at pH 7.0 in COVs reconstituted with mitochondrial proteins (Zazueta *et al.*, 1991). The six positive charges of this trinuclear complex $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]^{6+}Cl_6$ could account for nonspecific binding to negative charges of the phospholipids from COVs.

Selective Labeling of Membrane Proteins with the Inhibitor $^{103}Ru_{360}$

Ying and co-workers (1991) reported the isolation of a derivative of ruthenium red (Ru_{360}) with higher inhibitory activity than ruthenium red itself. Our approach was to synthesize the labeled ruthenium amine complex and use it as an affinity ligand for the calcium uniporter. A spectophotometric analysis was performed to evaluate the purity of the complex. As described, the binuclear complex elutes at 0.4 M of the formate linear gradient as a single peak that absorbs strongly at 355–360 nm. The recovery of the labeled compound was 140 nmol, with a specific activity of 587,000 cpm/nmol. The kinetics mode of action of the binuclear amine complex on mitochondrial calcium transport was measured by varying calcium concentration in the presence of different amounts of inhibitor. Figure 5 shows a Dixon plot of kinetics data of calcium transport obtained with rat kidney mitochondria. Typical noncompetitive inhibition curves were obtained. The K_i of Ru_{360} was 7.5 nM, which is in agreement with the inhibition rates obtained by Ying *et al.* (1991)

Table I. Enrichment of Calcium Uniporter Activity in COVs[a]

Solubilized extract reconstituted in COVs	Total protein (mg)	Specific activity (nmol Ca^{2+}/mg/5 min)	+ RR 5 μM (nmol Ca^{2+}/mg/5 min)	Yield (%)
Mitochondria	4000.00	151.80 ± 10.0	71.2 ± 10.0	100.0
Submitochondrial particles	1350.00	127.13 ± 13.0	63.2 ± 9.0	33.75
Ammonium sulfate (90%)	18.54	443.00 ± 32.0	251.3 ± 42.1	0.46
Immunoaffinity chromatography	4.90	1655.03 ± 408.12	1203 (2)	0.12
Liquid-phase isoelectrofocusing	0.20	14888.00 ± 2922.0	(n.e.)	0.01

[a] Mitochondrial fractions were incorporated in COVs (1.5–3.25 μg/mg phospholipids) and incubated in a medium containing 50 mM KH_2PO_4, 7.5 mM ascorbate, 0.75 mM TMPD, 150 μg cytochrome *c*, and 0.5 mM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). After 5 min, an aliquot was filtered through a 0.45 μm-Millipore filter and washed with 10 mM of $CaCl_2$. Specific activity values represent the difference between the energized and nonenergized uptake rates. In the indicated cases, ruthenium red (RR) 5 μM was used to inhibit the energized uptake. Final volume 1 ml; temperature 30°C; pH 7.0. n.e., not evaluated.

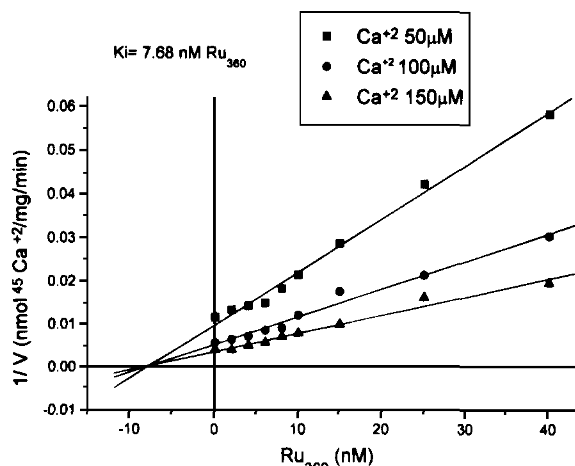

Fig. 5. Dixon plots of the kinetics data of mitochondrial Ca^{2+} transport in the presence of increasing concentrations of Ru_{360}. (■) At 50 μM Ca^{2+}; (●) 100 μM Ca^{2+}; and (▲) 150 μM Ca^{2+}. The medium was the same as in Fig. 4, except that $^{45}CaCl_2$ was used. Temperature, 25°C; pH 7.3.

by using this compound to inhibit Ca^{2+}-stimulated respiration of rat liver mitochondria.

The affinity of the binuclear complex was evaluated by equilibrium binding to intact mitochondria. As observed in Fig. 6, the binding at high concentrations of the inhibitor was not saturable. Indeed, the high-affinity sites obtained by a Scatchard plot, i.e., 11 pmol/mg protein, correlates with maximum levels of inhibition.

The affinity of the binuclear complex to mitochondrial proteins was also analyzed in submitochondrial particles labeled with 10 pmol $^{103}Ru_{360}$/mg protein by native and SDS-electrophoresis. In Fig. 7, the distribution of radioactivity in each 2-mm slice of a 12% SDS–polyacrylamide gel is shown, as well as the autoradiography of a gel run under the same conditions and exposed for several weeks. The assay clearly reveals a main labeled protein of low molecular weight. An approach to obtain this labeled protein was made by using preparative electroelution in liquid phase (Fig. 8). The protein that binds the labeled oxo-bridged ammonium complex elutes with other proteins of nearly the same molecular weight, i.e., 18 kDa, but the assay conditions limited the extraction of a single band.

Because of the cationic nature of Ru_{360}, (μ-O)[(HCO_2) $(NH_3)Ru_2$]Cl_3, it may simply interact with negatively charged groups on proteins. Thus, the specificity of the dye toward the calcium transporter was assayed by means of an isoelectric focusing separation of labeled proteins. The main radioactivity was detected on the range of acidic proteins, which is in good correspondence with the fraction that exerts maximal Ca^{2+} transport activity (not shown).

With the idea of obtaining the protein that binds $^{103}Ru_{360}$ with high selectivity, an "affinity" column was

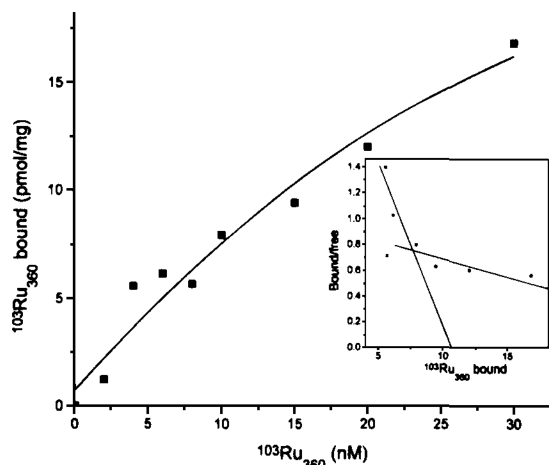

Fig. 6. Equilibrium binding of $^{103}Ru_{360}$ to intact mitochondria. The medium was the same as in Fig. 4, except that arsenazo III was not added. The insert shows the Scatchard plot analysis. Temperature, 25°C; pH 7.3.

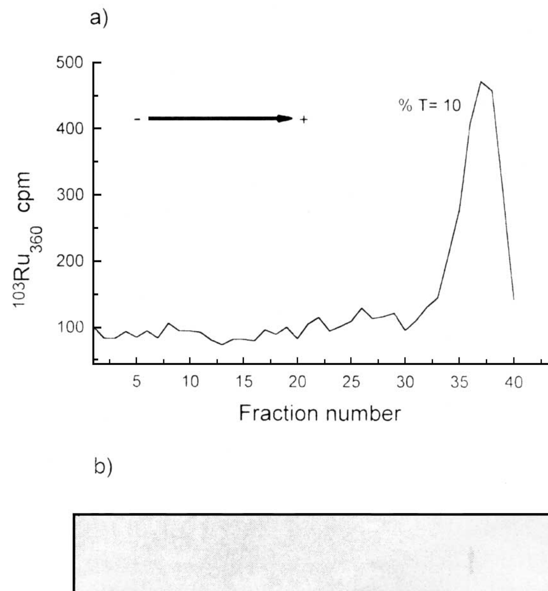

Fig. 7. (a) Radioactivity profile of SMP labeled with $^{103}Ru_{360}$, electrophoresed in a 12% SDS–polyacrylamide gel. Mitochondria (150 μg) or submitochondrial particles were labeled with 10 pmol/mg $^{103}Ru_{360}$, centrifuged, and resuspended in 2% of SDS under nonreducing conditions. The radioactivity in each 2-mm slice of the gel was counted in a γ-counter. (b) Proteins, separated under the same conditions, were dried and exposed to a X-ray film for nearly 12 weeks.

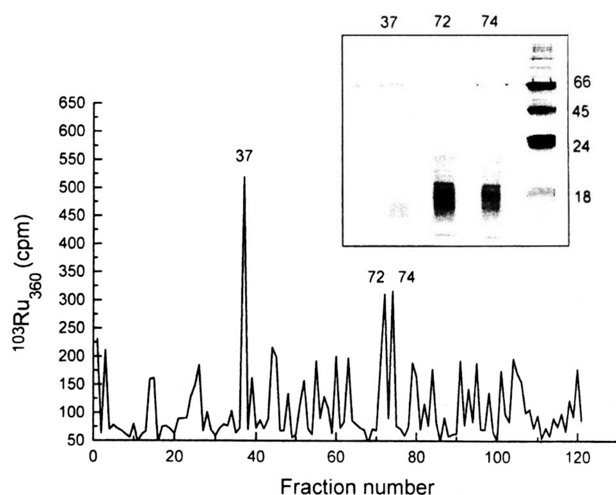

Fig. 8. Liquid-phase electroelution of submitochondrial particles labeled with 10 pmol/mg $^{103}Ru_{360}$. Submitochondrial particles were labeled as described in Fig. 5. Protein (2 mg) was resuspended in 2% SDS buffer and electrophoresed at constant power (12 W) until the dye front reached the end of the preparative 12% gel. The eluted fractions were recovered by increasing molecular weight order in 10 mM Tris, pH 7.0. The samples were concentrated and analyzed by silver stain in an analytical 12% SDS denaturing gel.

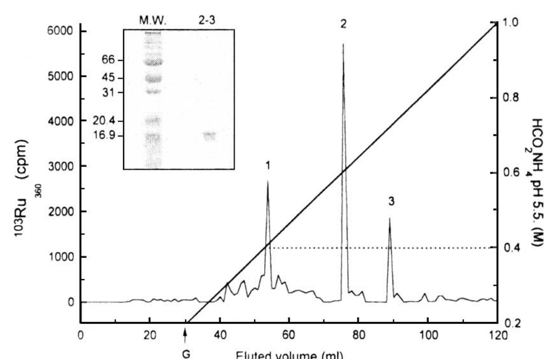

Fig. 9. Radioactivity profile of eluted submitochondrial proteins labeled with $^{103}Ru_{360}$ and separated by a linear ammonium formate gradient from a CM-cellulose matrix. Submitochondrial particles were labeled as described in Fig. 5. The pellet was solubilized in 1% $C_{12}E_8$ or 1% CHAPS and centrifuged at 100,000 × *g* for 1 h. The supernatant was loaded onto a CM-cellulose column equilibrated with 0.2 ammonium formate pH 5.5 and washed exhaustively with the same buffer. Where indicated, a linear gradient from 0.2 to 1.0 M was applied. In the insert the Coumassie-stained image of the proteins that retains the label is shown.

prepared by applying the assay for the separation of the ammonia complexes formed in the synthesis of ruthenium red and Ru_{360}. Solubilized membrane protein (20 mg) with 1% $C_{12}E_8$ or 1% CHAPS was incubated with 10 pmol/mg protein of $^{103}Ru_{360}$. The unbound inhibitor was eliminated by exhaustive dialysis against 0.2 M ammonium formate, pH 5.5. The labeled protein was loaded into a 15 × 5.5 cm CM-cellulose packed column in the presence of the same detergent concentration. After extensive washing with the same buffer, a linear gradient from 0.2 M to 1.0 ammonium formate, pH 5.5, was applied. Figure 9 shows the radioactivity profile of the eluted proteins. Three peaks were resolved; one of them represents unbound $^{103}Ru_{360}$, which elutes at 0.4 M of the gradient. It has not associated proteins as evaluated in a silver-stained SDS–polyacrylamide gel. The remaining two fractions were also analyzed by electrophoresis. Both presented a 18-kDa protein associated with small contaminants, detected by silver stain. These fractions were pooled and reanalyzed by electrophoresis; the Coumassie-stained image is shown in Fig. 9 (insert).

DISCUSSION

Early attempts to purify the calcium uniporter included the isolation of factors that bind Ca^{2+} with high affinity (Lenhinger, 1971; Gómez–Puyou *et al.*, 1972). The discovery by Moore (1971) that ruthenium red is a potent inhibitor of calcium uptake drove the search toward carbohydrate-containing components in mitochondria that possessed the ability to bind Ca^{2+}. A considerable effort has been expended toward the extraction of a mitochondrial glycoprotein with Ca^{2+}-binding or transport activity. The most extensively studied are a set of glycoproteins (33,000–42,000 kDa), which appear to be located in the intermembrane space and to be removed by hypotonic rupture of the outer membrane (Sottocasa *et al.*, 1971, 1972; Sandri *et al.*, 1976; Prestipino *et al.*, 1974). They bind calcium with both high and low affinity and this binding is inhibited by ruthenium red and lanthanides. The rate of calcium uptake is lowered in preparations from which the glycoprotein has been removed and is partially restored upon addition to the suspension. Antibodies to the glycoprotein significantly inhibit Ca^{2+} uptake. The lack of sensitivity of this protein to agents that dissipate the membrane potential generated by respiration and the fact that it was easily extracted by osmotic shock indicated that this protein was located in the intermembranal space or loosely bound to the membrane, so its role on the uptake mechanism was suggested as a superficial site of recognition for calcium.

Recently, Mironova and co-workers (1994) reported the purification of a low-molecular-weight peptide from rat liver mitochondria that formed selec-

tive channels. These channels were strongly inhibited by 1–4 μM ruthenium red. This observation led to that group to suggest that the channel is a constituent of the mitochondrial calcium uniporter.

In this paper, we describe a fractionation procedure for enriching calcium uniporter proteins from rat kidney submitochondrial particles. Important steps in our purification scheme were the enrichment of the fraction, obtained by salt precipitation, followed by liquid-phase isoelectrofocusing in the presence of a zwitterionic detergent and protective agents, such as glycerol. The data presented show that the calcium transporting fraction incorporated into liposomes exhibits properties that are distinctive features of the Ca^{2+} uniporter in mitochondria, such as the absolute requirement of a negative inner membrane potential that drives the accumulation of the cation and the inhibitor sensitivity.

Isolation of the mitochondrial proteins responsible for transport of cations across the inner membrane is an arduous task. With the exception of the uncoupling protein (Strieleman *et al.*, 1985; Casteilla *et al.*, 1990; Jezek, *et al.*, 1990), the mitochondrial cation transporting proteins have not been purified and their cDNAs have not been cloned. Other cation mitochondrial transporters, e.g., the Na^+/Ca^{2+} antiporter, the K^+/H^+ exchanger, and the Na^+-selective Na^+/H^+ antiporter, have been only partially purified and reconstituted (Li *et al.*, 1990, 1992; Garlid *et al.*, 1991)

The use of specific ligands, providing the means for the identification of a particular protein, is of special interest, since the isolation and reconstitution process would otherwise be a lengthy and tedious technical procedure. The inhibitor Ru_{360} has the characteristics of a satisfactory affinity ligand for purification of the uniporter. It has a very high affinity for the calcium uniporter, as the binding sites associated with the maximum inhibition rates correspond to 11 pmol/mg protein in whole mitochondria. The reported maximum specific binding for this compound is 7.5 pmol/mg protein (Ying *et al.*, 1991). The type of inhibition is noncompetitive with Ca^{2+}. Our results indicate that the protein that binds the oxo-bridged binuclear compound is related to the 18 kDa protein extracted from rat kidney mitochondria and reconstituted in cytochrome oxidase vesicles.

There are no reports concerning the affinity of this compound toward Ca^{2+}-binding glycoproteins, so we cannot discard the glycoside nature of the isolated protein. However, the fact that it is located in the inner mitochondrial membrane suggested that this protein is different from the set of glycoproteins already mentioned.

It should be noted that under nondenaturing electrophoresis analysis, we found only one band from the isoelectrofocused fractions with Ca^{2+} transport activity; however, this preparation showed two subunits of 18 and 70 kDa in SDS–polyacrylamide electrophoresis. The subunit of 70 kDa is only detectable by silver stain; it may be a feature of the technique or a minor contaminant. We suggest that both could be part of a multicomponent system, in addition to a soluble recognition site located in the intermembrane space represented by the multicited glycoprotein. From our data, the arrangement of such a complex could only be speculated—it could resemble the structure of the Na^+ channel that consists of a single polypeptide chain with repeating units (Noda *et al.*, 1986). If the transporting protein is represented by the 70-kDa subunit, the role of the minor subunit would be only that of an inhibitor binding site.

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