Journal of Bioenergetics and Biomembranes, Vol. 36, No. 5, October 2004 (© 2004)

Different Subunit Location of the Inhibition and Transport Sites in the Mitochondrial Calcium Uniporter

C. Zazueta,[1,2] F. Correa,[1] N. García,[1] and G. de J. García[1]

Received June 7, 2004; accepted June 20, 2004

The mitochondrial calcium uniporter behaves as a cooperative mechanism, where the velocity is dependent on $[Ca^{2+}]_{ex}$. Transport kinetics follows a sigmoidal behavior with a Hill coefficient near 2.0, indicating the binding of at least two calcium molecules. Calcium transport in mitochondria is dependent on a negative inner membrane potential and is inhibited by policationic ruthenium compounds. In this study, calcium uptake activity was reconstituted into cytochrome oxidase vesicles by incorporating solubilized mitochondrial proteins. Calcium accumulation plotted against increasing Ca^{2+} concentrations followed a sigmoidal behavior with a Hill coefficient of 1.53. The uptake was sensitive to ruthenium policationic inhibitors, e.g. ruthenium red and Ru_{360}. After mitochondrial proteins were separated by preparative isoelectrofocusing and incorporated into cytochrome oxidase vesicles, two peaks of calcium uptake activity were recovered. One of the activities was inhibited by Ru_{360}, while the second activity was insensitive to Ru_{360} and was associated with proteins focused at very acidic isoelectric points. By using a thiol-group crosslinker and radiolabeled Ru_{360}, we proposed a scheme of partial dissociation of the uniporter inhibitor-binding subunit under acidic conditions.

KEY WORDS: Calcium; isoelectrofocusing; mitochondria; pH; uniporter.

INTRODUCTION

The mitochondrial calcium uptake mechanism is a cooperative system with sigmoidal kinetics and a Hill number between 1.7–2.0 (Gunter and Pfeiffer, 1990). At low Ca^{2+} and Pr^{3+} concentrations, calcium accumulation loses its sigmoidal character, this led Kröner (1986) to suggest a model of two different sites for calcium binding to the uniporter molecule, one site for activation and a second site for transport. By the same token, reverse calcium uptake activity was measured in the presence of CCCP in calcium preloaded mitochondria by Igvabvoa and Pfeiffer (1991); under these conditions, EGTA inhibited the uncoupler-induced calcium release in mitochondria, suggesting an action on the regulatory site in the calcium uniporter. They also proposed that the regulatory site would be an intermembrane component, as EGTA did not diminish the reverse calcium uptake in mitoplasts.

It has also been reported that reconstituted calcium transport with semipurified mitochondrial proteins, shows a hyperbolic behavior (Garlid, 1994). These results could be explained in terms of a multimeric model for the uniporter, where one of the putative calcium binding sites at the uniporter is lost during the purification procedure. There is no available molecular information about the calcium uniporter, the current knowledge about this transporter is derived only from biochemical studies. Nevertheless, we have isolated an 18-kDa protein that binds Ru_{360} with high affinity (Zazueta *et al*., 1998). We proposed that this protein could be a component of the mitochondrial calcium uniporter. In this work we show results that suggest that the calcium uniporter structure could be oligomeric

[1] Departamento de Bioquímica, Instituto Nacional de Cardiología Ignacio Chávez, México, D. F., Mexico.

[2] To whom correspondence should be addressed at Instituto Nacional de Cardiología, Departamento de Bioquímica, Juan Badiano No. 1, Colonia Sección XVI, 14080 México, D. F., México; e-mail: czazuetam@hotmail.com

Key to abbreviations: CCCP, Carbonyl-cyanide m-chlorophenylhydrazone; EGTA, Ethylene glycol-bis (2-aminoethyleter)-N,N,N′,N′-tetraacetic acid; CHAPS, 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonate; COV, Cytochrome oxidase vesicles; TRIS, Tris(Hydroxymethyl) aminomethane; DIDS, 4-4′-Diisothiochyanate stilbene-2-2′ disodium salt.

and that the low molecular weight protein already mentioned contains the inhibitor binding site.

MATERIAL AND METHODS

Beef heart mitochondria were obtained as previously described (Chávez *et al*., 1985). Submitochondrial particles (SMP) were obtained by the method of Lee and Ernster (1965). SMP (3 mg/mL) were solubilized with 1.2% sodium cholate (w/v) in a medium containing 250 mM sucrose, 10 mM TRIS, pH 7.3. Protein extraction was carried out with constant stirring for 30 min at 4°C. The suspension was centrifuged for 1 h at 100,000 × *g*. Extracted proteins were saturated with 50% ammonium sulfate and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant containing 8 mg protein/mL, was dialyzed against 10 mM TRIS, pH 7.0 and passed through an ion retardation desalting column. The sample was diluted to a final volume of 55 mL, containing 0.5% CHAPS (w/v), 20% glycerol, and 2 mL of ByoLyte ampholytes (BIORAD Laboratories, Inc., Hercules, CA), pH range 5–8. This solution was loaded into a liquid-phase isoelectrofocusing Rotofor Cell (BIORAD Laboratories, Inc., Hercules, CA) without further treatment. Focusing was carried out at 12 W constant power for 5–6 h at 4°C. At equilibrium the values were around 690 V and V/H = 2403. Twenty fractions were harvested and their pH values were measured. Protein concentration was determined by a modified Lowry procedure (Nakamura *et al*., 1983). Reconstitution of liquid-phase isoelectrofocused proteins and determination of calcium uptake activity was evaluated as previously described (Zazueta *et al*., 1998). Briefly, dried lipids (30 mg asolectin/mL) were sonicated to clarity in 50 mM H_3PO_4-TEA (triethanolamine) pH 7.0. Cytochrome oxidase was added to a final concentration of 0.25 mg/mL and incorporated by simple mixing with the liposomes. Total mitochondrial proteins were solubilized with 1.2% sodium cholate and incorporated into cytochrome oxidase vesicles (COV) after exhaustive dialysis. Isoelectrofocused proteins were incubated with 1 M NaCl (final concentration) and dialyzed against 250 volumes of 50 mM KH_2PO_4, pH 7.0 to eliminate ampholytes, except in refocusing experiments. Each fraction (60–90 μg) was incubated with 0.5 mL COV and sonicated in a water sonicator for 5 s in the presence of 0.5% sodium cholate. The detergent was removed by dilution with 40 volumes of 50 mM KH_2PO_4, pH 7.0 and concentrated overnight with Aquazide II (Calbiochem, San Diego, CA). Incorporated proteins in COVs were added to an incubation medium containing 50 mM KH_2PO_4, pH 7.0, 7.5 mM ascorbate, 0.75 mM TMPD (tetramethyl-1-phenylendiamine), 150 μg cytochrome *c* 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). Activity results are representative of three independent experiments with similar results. For crosslinking experiments, diisothiocyanate stilbene (DIDS) was incubated in the dark for 15–30 min at 4°C with the ammonium sulfate supernatant. Before preparative isoelectrofocusing fractionation, the samples were desalted by using G-10 (BIORAD Laboratories, Inc., Hercules, CA) ion-retarding columns. Radioactive Ru_{360} was synthesized according to the method described by Ying *et al*. (1991). Binding of $^{103}Ru_{360}$ complex to isoelectrofocused proteins was accomplished by incubating 100 nM of the labeled complex (specific activity 50 cpm/pmol) for 1 min. The samples were electrophoresed under denaturing and reducing conditions (Laemmli, 1970) in a gradient polyacrylamide gel (12–15%). Duplicated gels were run under the same conditions for protein silver stain. After electrophoresis, the gels were measured and the individual lines separated and cut into 4 mm slices to evaluate the radiolabel location and compared against the stained gels.

RESULTS

Reconstituted calcium transport was measured in cytochrome oxidase vesicles with incorporated solubilized mitochondrial proteins at different calcium concentrations, (Fig. 1). Calcium uptake showed a cooperative

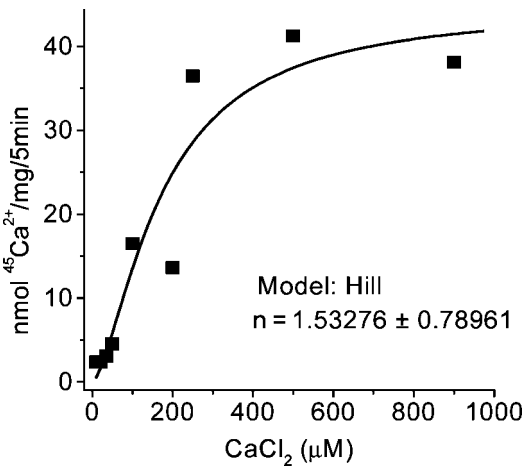

Fig. 1. Reconstituted calcium uptake into COVs, evaluated at different external calcium concentrations. Data were fitted to the Hill model by using the Microcal Origin™ program from Microcal Software Inc. (Northampton, MA), according to the equation $v/V_{max} = [S]^n/K_s + [S]^n$; where n = number of substrate binding sites per molecule of enzyme and K_s = dissociation constant.

behavior, as deduced from the Hill analysis that yields a value of $n = 1.53$. The transport was inhibited by ruthenium red and Ru_{360} (data not shown), so we assumed that the extraction and reconstitution procedures did not affect the basic structure of the mitochondrial calcium uniporter.

Figure 2 shows the preparative isoelectrofocusing separation of the uniporter enriched fraction. Panel A, shows the pH gradient obtained after the system reached equilibrium. Twenty fractions were obtained and incorporated into COV. Aliquots of the electrofocused fractions were withdrawn and incubated with Ru_{360} before their incorporation into COV and activity analysis. Calcium transport activity was related to proteins showing isoelectric points (Ips) between 1.5–2.0 and nearly 5.0

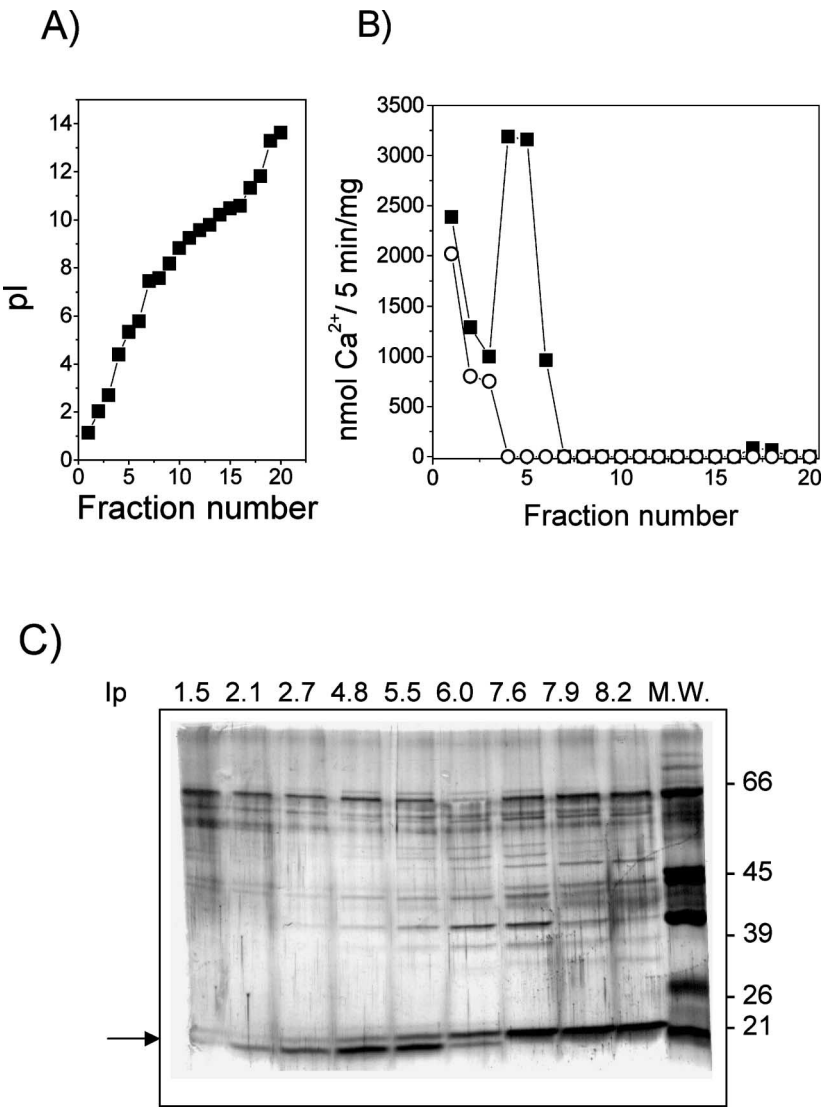

Fig. 2. Fractionating of mitochondrial proteins by liquid-phase isoelectrofocusing. (A) pH gradient formed after isoelectrofocusing at 12 W constant power for 5–6 h at 4°C. (B) Calcium uptake specific activity measured in COVs reconstituted with isoelectrofocused proteins. Aliquots of the 20-fractions obtained after isoelectrofocusing were reconstituted into COVs and evaluated for calcium uptake activity as described. (■) Aliquots without further treatment; (○) Aliquots incubated with 40 nM Ru_{360} prior to reconstitution. (C) Silver protein stain of fractions showing calcium uptake activity. The silver stained gel is representative of two independent experiments with similar results.

(panel B). The proteins with lower Ips, lost the ability to recognize the dinuclear inhibitor Ru_{360}, but still maintained calcium uptake activity, although the activity was diminished presumably by pH inactivation. The proteins present in these fractions were analyzed by denaturing gel electrophoresis (panel C). The samples with activity and inhibitor sensitivity contain, among others, an 18-kDa protein presumably related with the one that we have demonstrated that binds the inhibitor $^{103}Ru_{360}$ (Zazueta *et al.*, 1998). To ascertain that the inhibitor-sensitive component of the uniporter was the one dissociated under acidic conditions, we refocused the samples that maintained activity and inhibitor sensitivity after the isoelectrofocusing treatment (Ips around 5.0). As the samples already contained the ampholytes of that particular pH, the new pH gradient was extended in the range between 5.0 and 6.0 (Fig. 3,

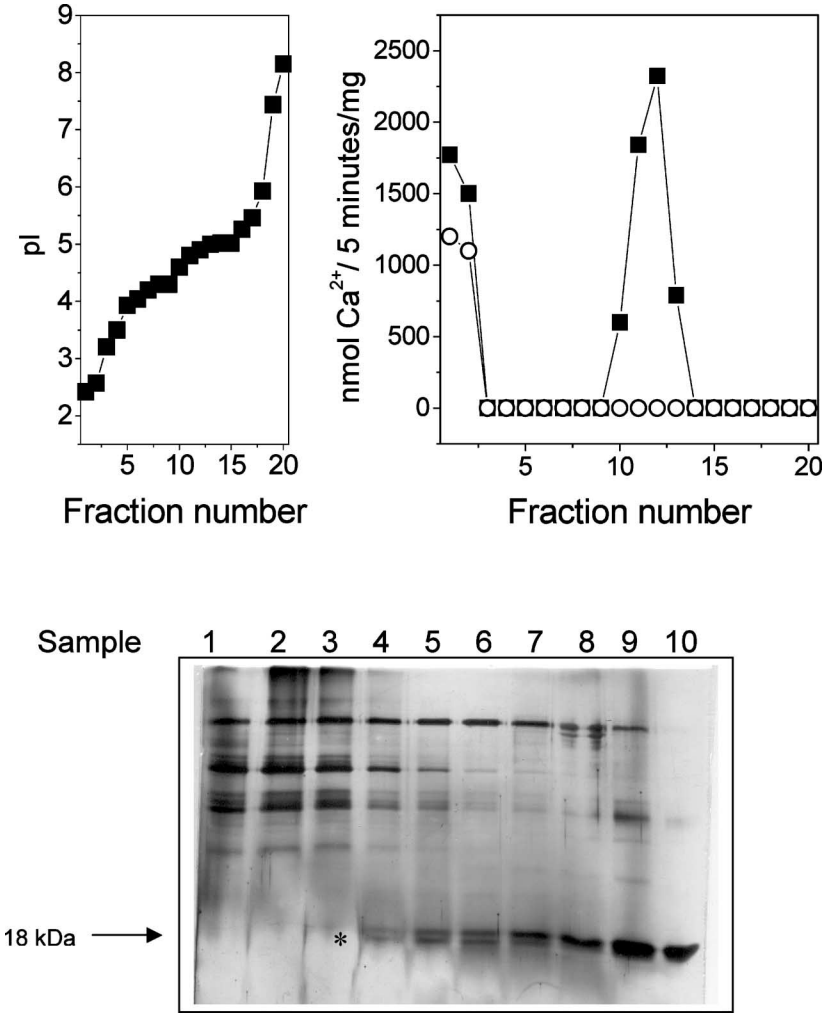

Fig. 3. Re-isoelectrofocusing of mitochondrial proteins with Ips between 4.8–5.5. Samples containing ampholytes between pH 4.8 and 5.5 were pooled and isoelectrofocused at 12 W constant power, until the system reached the equilibrium (constant voltage). (A) pH gradient formed after isoelectrofocusing at 12 W constant power at 4°C. (B) Calcium uptake specific activity measured in COVs reconstituted with isoelectrofocused proteins. Aliquots of the 20-fractions obtained after re-isoelectrofocusing were reconstituted into COVs and evaluated for calcium uptake activity as described. (■) Aliquots without further treatment; (○) Aliquots incubated with 40 nM Ru_{360} prior to reconstitution. (C) Silver protein stain of fractions showing calcium uptake activity. Samples 1–3, correspond to Ips = 2.4–3.2; samples 4–7, to Ips = 4.5–5.0; and samples 8–10, to Ips = 6.0–8.2. The silver stained gel is representative of two independent experiments with similar results.

panel A). The twenty generated fractions were reconstituted in COV and evaluated for potential calcium uptake. Two activities were detected that corresponded to proteins of Ips of 2.5 and proteins with Ips around 5.0. Only the latter was inhibited by 40 nM Ru_{360} (panel B). Taken together, these results suggest that the channel-forming subunit can be partially dissociated from a different subunit that possesses the inhibitor binding site.

Because it has been suggested that dithiol groups are essential components of the mitochondrial calcium uniporter (Chávez *et al*., 1985), we used the crosslinker agent diisothiocyanate stilbene (DIDS) to protect the subunit dissociation achieved under acidic conditions. Solubilized mitochondrial proteins were incubated in a phosphate medium containing 200 μM DIDS and the reaction progress was followed by spectrophotometry at 340 nm (Fig. 4). Controls without protein showed constant absorbance measurements at 340 nm for almost 60 min, while with the crosslinker agent, absorbance clearly diminished when protein was present in the incubation medium. Reaction constant rates were obtained from a semilogarithmic plot of absorbance percentage against time (Fig. 4, insert A). DIDS absorbance diminished at a constant rate of 0.004 min^{-1} in a medium without protein versus 0.001 min^{-1} in samples containing proteins. Crosslinked proteins were isoelectrofocused and a polyacrylamide gel electrophoresis analysis of the fractions showing calcium transport activity and inhibitor

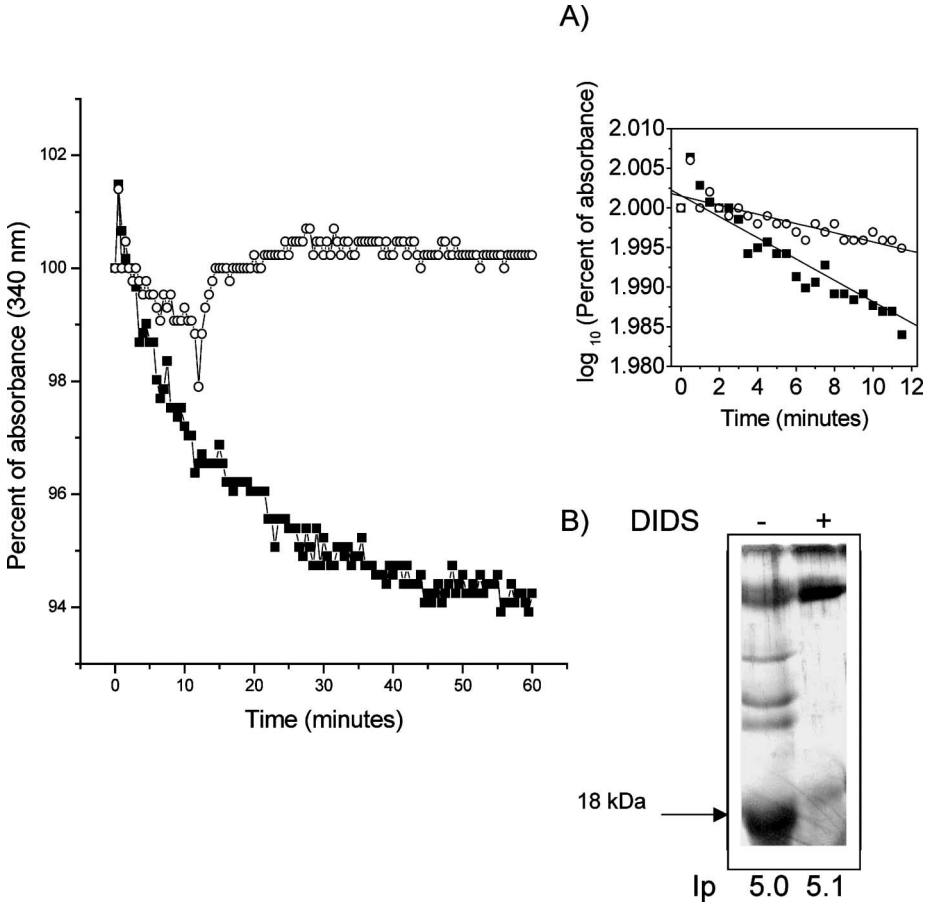

Fig. 4. Crosslinking of mitochondrial proteins in the presence of DIDS. Percent change of absorbance at 340 nm in samples without protein (○) versus solubilized mitochondrial proteins incubated with 200 μM DIDS in 50 mM KH_2PO_4, pH 7.0 (■). The semilogarithmic plot of the velocity curve is shown in the upper inset. The silver stained pattern of crosslinked proteins after isoelectrofocusing procedure is also shown. Crosslinked proteins were subjected to liquid phase-chromatography. After isoelectrofocusing, proteins were incubated with NaCl to a final concentration of 1 M to eliminate the ampholytes and passed through desalting G-10 columns (BIORAD Laboratories, Inc., Hercules, CA). Fractions with Ip = 5.0 were compared with control proteins focused at Ips near 5.0. The silver stained gel is representative of two independent experiments with similar results.

sensitivity was performed (Ips around 5.0). Silver staining of the electrophoresed proteins without DIDS, show a major 18-kDa protein, that decreases remarkably in the presence of DIDS (Fig. 4, insert B). Thus, we can conclude that the small subunit contains thiol residues that could make a bridge with the two partially positives atoms of the thiocyanate group from DIDS, according to the mechanism proposed by Bernardes *et al.* (1994). When calcium transport activity was measured in reconstituted COV, using control and crosslinked proteins with isoelectric points around 5.0, there was no calcium accumulation into the vesicles reconstituted with crosslinked proteins (not shown). Evidences suggest that DIDS interacts with inner mitochondrial membrane proteins (Bernardes *et al.*, 1994) and that it inhibits calcium transport in *Trypanosoma cruzi* (Bernardes *et al.*, 2000). Indeed, the possibility exists that, under crosslinking conditions the uniporter molecule could be associated with other proteins, changing its net charge and being redistributed at different Ips with the isoelectrofocusing procedure. To test this hypothesis, $^{103}Ru_{360}$ binding experiments with the crosslinked proteins were performed. Proteins that focused at pH 5.0 were incubated with 100 nM of the labeled compound and separated by electrophoresis, maximum peaks of radioactivity were located in low molecular weight proteins. Crosslinked proteins recovered at the same isoelectric point (Ip = 5.1) were also incubated in the presence of $^{103}Ru_{360}$. At such pH, the radioactive label was displaced to high molecular weight proteins (Fig. 5). Furthermore, label displayed an unspecific pattern as it was also found associated with proteins focused at different isoelectric points (not shown). Taking the results obtained by the fractionation analysis together with the findings of the binding assays, we conclude that the calcium uniporter is partially dissociated under acidic conditions, in such a way that the inhibitor binding subunit is lost. By this approach we propose that the calcium uniporter is constituted of at least two different subunits.

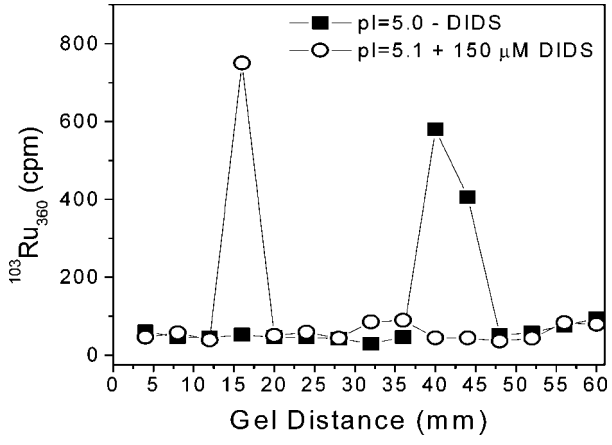

Fig. 5. Crosslinked proteins and control proteins with Ips = 5.0 were incubated with 40 nM $^{103}Ru_{360}$ and analyzed by SDS-PAGE. Both lines were cut into 4 mm slices and measured for radioactivity. Control proteins (○) versus crosslinked proteins (■).

DISCUSSION

The mitochondrial calcium uniporter is a fast "gated" pore, that contains a calcium cytosolic binding site in the inner membrane that activates the transport (Litsky and Pfeiffer, 1997; Sparagna *et al.*, 1995). It has been suggested that the calcium uniporter contains at least two subunits, one of them a dissociable factor related to glycoproteins (Igvabvoa and Pfeiffer, 1991). This group also proposed that the uniporter is regulated by association–dissociation cycles of this factor, activated by calcium binding.

In this work, we reconstituted calcium transport in cytochrome oxidase vesicles which had extracted mitochondrial proteins incorporated. Reconstituted transport was sigmoidal with a Hill coefficient close to that reported in intact mitochondria, i.e. 1.7–2.0 (Gunter and Pfeiffer, 1990) and was inhibited by ruthenium red and Ru_{360}. This oxo-bridged polynuclear compound is by now the most potent and specific inhibitor of mitochondrial calcium uptake (Matlib *et al.*, 1998; Ying *et al.*, 1991; Zazueta *et al.*, 1999). Such property led our group to identify an 18-kDa mitochondrial protein with high affinity for this compound (Zazueta *et al.*, 1998). We suggested then, that this entity and a major transporting protein could form a multicomponent system. Analysis of the calcium uniporter transport appears to be consistent with an ion channel rather than with another transport mechanism. Recently, a novel mitochondrial ion channel that binds Ca^{2+} with extremely high affinity has been identified (Kirichok *et al.*, 2004). It is conceivable that like other cation channels (Catterall, 1995; Hartshorne *et al.*, 1985), the mitochondrial calcium uniporter could be a heteromeric membrane protein. Whether the Ru_{360} binding site is located in the uniporter protein or in a regulatory protein molecule associated with the uniporter is under debate. Some groups argue that this issue would not be determined until the uniporter protein or the Ru_{360} binding site is identified (Matlib *et al.*, 1998). Indeed, by using a liquid phase isoelectrofocusing fractionating model, we achieved the dissociation of transporting and inhibitor-binding subunits from the calcium uniporter. This model is based on the tenet that pH modification, among other conditions, induces protein subunit dissociation (Chang *et al.*, 1997; Syto *et al.*, 1998). On the

basis of the results of this paper, we propose that the calcium uniporter is composed of at least two different subunits, that become partially dissociated at low pHs. The inhibitor-resistant proteins are dissociated at low pH and represent the calcium channel, the inhibitor-binding subunit remains linked to the channel at higher pH. Furthermore, this small subunit appears to contain thiol residues that are presumably bridged with the two partially positive atoms of the thiocyanate group from DIDS, according to the mechanism proposed by Bernardes *et al*. (1994). In this respect, early studies with cadmium suggested that sulphydryl groups are relevant for the activity of the mitochondrial calcium uniporter, as dithiothreitol reverted the inhibitory effect of cadmium on calcium uptake (Chávez *et al*., 1985). The present study also shows that calcium transport is abolished in presence of DIDS. This finding is consistent with recent evidence that DIDS inhibits, in a dose-dependent manner, the calcium transport in *Trypanosoma cruzi* mitochondria (Bernardes *et al*., 2000).

ACKNOWLEDGMENT

This work received partial support from grant 34387-N from National Council of Science and Technology to CZ.

REFERENCES

Bernardes, C., Meyer-Fernandés, J., Basseres, D., Castilho, R., and Vercesi, A. (1994). *Biochim. Biophys. Acta* **1188**, 93–100.

Bernardes, C., Meyer-Fernandez, J., Saad-Nehme, J., Vannier-Santos, M., Peres-Sampaio, C., and Vercesi, A. (2000). *Int. J. Biochem. Cell Biol.* **32**, 519–527.

Catterall, W. (1995). *Annu. Rev. Biochem.* **64**, 493–531.

Chang, G., Lee, H., and Chow, R. (1997). *Exp. Eye Res.* **65**, 653–659.

Chávez, E., Briones, R., Michel, B., Bravo, C., and Jay, D. (1985). *Arch. Biochem. Biophys.* **242**, 493–497.

Garlid, K. (1994). *J. Bioenerg. Biomembr.* **26**, 537–542.

Gunter, T., and Pfeiffer, D. (1990). *Am. J. Physiol.* **258**,C755–C786.

Hartshorne, R., Keller, B., Talvenheimo, J., Catterall, W., and Montal, M. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82**, 240–244.

Igvabvoa, I., and Pfeiffer, D. (1991). *J. Biol. Chem.* **266**, 4283–4287.

Kirichok, Y., Krapivinsky, G., and Clapham, D. (2004). *Nature* **427**, 360–364.

Kröner, H. (1986). *Arch. Biochem. Biophys.* **251**, 525–535.

Laemmli, U. (1970). *Nature* **277**, 680–685.

Lee, C. P., and Ernster, J. (1965). In *Symposium on the Regulation of Metabolic Process in Mitochondria*, Vol. **7** (Tager, J., Papa, S., Quagliariello, E., and Slater, E., eds.), Elsevier/North Holland, New York, pp. 218–234.

Litsky, M., and Pfeiffer, D. (1997). *Biochemistry* **38**, 7071–7080.

Matlib, M., Zhou, Z., Knight, S., Ahmed, S., Choi, K., Krause-Bauer, J., Phillips, J., Altschud, R., Katsube, S., Sperelakis, N., and Bers, D. (1998). *J. Biol. Chem.* **273**, 10223–10231.

Nakamura, J., Wang, T., Tsai, L., and Schwartz, A. (1983). *J. Biol. Chem.* **258**, 5079–5083.

Rosier, R., Gunter, T., Tucker, D., and Gunter, K. (1979). *Anal. Biochem.* **96**, 384–390.

Sparagna, G., Gunter, K., Sheu, S., and Gunter, T. (1995). *J. Biol. Chem.* **270**, 27510–27515.

Syto, R., Murgolo, N., Braswell, E., Mui, P., Huang, E., and Windsor, W. (1998). *Biochemistry* **37**, 16943–16951.

Ying, W., Emerson, J., Clarke, M., and Sanadi, D. (1991). *Biochemistry* **30**, 4949–4952.

Zazueta, C., Sosa-Torres, M., Correa, F., and Garza-Ortíz, A. (1999). *J. Bioenerg. Biomembr.* **31**, 551–557.

Zazueta, C., Zafra, G., Vera, G., Sánchez, C., and Chávez, E. (1998). *J. Bioenerg. Biomembr*. **30**, 489–498.