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Mitochondrial glycosidic residues contribute to the interaction between ruthenium amine complexes and the calcium uniporter

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Abstract

The role of glycosidic residues in the inhibitory properties of ruthenium complexes on mitochondrial calcium uptake was determined in mitoplasts.

Our results showed that the binding and inhibitory properties of ruthenium amine complexes were modified when mitoplasts were exposed to N-glycosidase F action, but calcium uptake was not altered. N-linked proteins of the mitochondrial inner membrane were identified. We detected an 18-kDa protein that binds labeled Ru_{360} under control conditions, but failed to bind the inhibitor after deglycosilation. A relationship between this protein and the action of ruthenium amine inhibitors of the mitochondrial uniporter is proposed. (Mol Cell Biochem **272**: 55–62, 2005)

Key words: calcium uniporter, glycoproteins, mitochondria, ruthenium complex inhibitors

Introduction

Considerable evidence has accumulated which indicates that glycoproteins are components of mitochondria [1–3]. Particularly, it has been suggested that the calcium transporter contains one or more glycoprotein subunits [1, 4–7]. This idea is supported by the fact that the proteoglycan dye ruthenium red inhibits it in the (sub)micromolar range [8–10]. Hexamine cobatilchloride, a selective reagent for the anionic groups of mucopolysaccharides, also abolishes mitochondrial calcium uptake with a half-maximum inhibition in the micromolar range [11]. In this respect, Mironova *et al*. [6] proposed a 40-kDa glycoprotein as the calcium uniporter. Later, they reported that such protein was a cytosolic contaminant related to the glycopeptide orosomucoid [7].

Among the mitochondrial electrophoretic cation flux catalysts, the first recognized and most studied system is the calcium uniporter. This transporter exhibits high affinity for its substrate [12], has a large transport capacity (>600 nmol Ca^{2+}/mg of protein/min), is inhibited by low concentrations of ruthenium red and Ru_{360} [13] and it has been identified in a large variety of organisms [14]. To determine if there is a relationship between the specific binding of ruthenium inhibitors to a glycosidic component in this transporter, we investigated the effect that specific glycosidases have on calcium accumulation and on the inhibitory properties of ruthenium red and Ru_{360} in mitoplasts obtained from purified mitochondria.

Previous studies from our own group suggested that an accessory 18-kDa mitochondrial protein, containing the inhibitor binding site, could be part of the mitochondrial uniporter. This proposal arose from the $^{103}Ru_{360}$ high specificity binding to that entity [15].

In this report we showed that ruthenium red and Ru_{360} bind a 18-kDa protein under control conditions, but no after deglycosilation. We also observed a dramatic change in

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the inhibitory properties of both compounds when mitoplasts were exposed to glycosidase F action.

The relevance of glycosidic residues for the proper binding of ruthenium complexes to the uniporter molecule and the inhibitor-protein interaction of these compounds is discussed.

Material and methods

N-glycosidase F and Digoxigenin-Glycan (DIG-Glycan) Protein kit were purchased from Roche Applied Science (Indianapolis, IN), $^{45}CaCl_2$ and $^{103}RuCl_3$ were obtained from NEN Life Science Products (Boston, MA). All other reagents were of analytical grade. The experiments were approved by the National Institute of Cardiology Animal Care and Use Committee.

Purification of mitochondria

Rat kidney mitochondria were obtained by differential centrifugation in a medium containing 250 mM sucrose, 10 mM TRIS and 1 mM EDTA, pH 7.3 as described [16]. Approximately 80 mg of protein in 1 ml were layered onto a discontinuous Percoll density gradient (25%–40%) and centrifuged at 36,000 × g for 15 min. Mitochondria populations were collected and washed with 250 mM sucrose, 10 mM TRIS, pH 7.3. Fractions containing mitochondria were centrifuged at 15,000 × g for 10 min and suspended in the same buffer.

Organelle marker enzyme determinations

Mitochondrial populations were evaluated for purity by measuring contaminant enzymatic marker activities for different organelles: acid phosphatase EC [3.1.3.2.] for lysosomes [17]; alkaline phosphatase EC [3.1.3.1] for plasma membranes [18]; D-glucose 6 phosphate phosphohydrolase EC [3.1.3.9] for endoplasmic reticulum [18]; catalase EC [1.1.1.6] for peroxisomes [19] and cytochrome oxidase EC [1.9.3.1] for mitochondria [20].

Preparation of mitoplasts

Digitonin dissolved in sucrose medium was slowly added to purified mitochondria to a final concentration of 0.07 mg digitonin/mg protein. The volume of added digitonin was equal to the volume of mitochondrial suspension. Outer membranes were dissolved by gentle stirring for 15 min at 4 °C and then diluted with three volumes of sucrose medium. Mitoplasts were obtained by centrifuging at 15,000 × g and then resuspending in the same buffer.

Glycosidase digestions

To hydrolyze all types of N-glycan chains, mitoplasts (0.05 mg) were incubated with a 10 μl solution containing 0.01 units of N-glycosidase F for 30 min at 30 °C. The specifications of the enzyme guaranteed the absence of the following contaminants: endoglycosidase F, β-galactosidase, β-glucosidase, α- and β-mannosidase, β-N-acetylhexosaminidase, α-L-fucosidase, sialidase and proteases.

The composition of the incubation buffer was 25 mM potassium phosphate, 10 mM EDTA, 2% Triton X-100, 0.2% SDS (w/v) and 1% 2-β mercaptoethanol (v/v), pH 8.0. At the end of the digestion, mitoplasts were examined by SDS-PAGE, followed by immunodetection of glycosilated proteins.

Immunodetection of glycosilated proteins

Sugar-labeling of glycoproteins in each fraction was carried out by using digoxigenin, according to DIG-Glycan (Digoxigenin-Glycan) Protein kit from Roche Applied Science (Indianapolis, IN). The technique involves the labeling of sugar side-chains, by specific oxidation of the sugar hydroxyl groups to aldehydes with periodate, followed by covalent coupling of the digoxigenin-hapten to the aldehyde groups created. Labeled samples were electrophoresed and blotted onto nitrocellulose sheets. Membranes were washed in 0.05 M potassium phosphate, pH 8.5 and incubated for 1 h against anti-DIG-alkaline phosphatase (AP) in TRIS-saline buffer (TBS). Detection was performed by incubating the alkaline phosphate system with an oxydizable substrate for the enzyme.

Calcium transport in deglycosilated mitoplasts

Mitoplasts used for calcium transport measurements were suspended in a medium containing 250 mM sucrose, 10 mM TRIS, 200 μM ADP, 1 μM cyclosporin A (CSA), 3 μM oligomycin, pH 7.3 and deglycosilated under mild conditions. Aliquots of 100 μg were withdrawn and incubated with 0.02 U N-glycosidase F dissolved in 50 mM sodium phosphate, 25 mM EDTA, 50% glycerol, pH 7.3 at ratio (1:1 volume ratio) for 30 min at room temperature. Mitoplasts were centrifuged at 15,000 × g and resuspended in 250 mM sucrose, 10 mM TRIS, pH 7.3.

Calcium uptake was measured as follows: 0.1 mg of mitoplast protein were incubated in 1 ml of 250 mM sucrose, 10 mM HEPES, 10 mM succinate, 3 mM Pi, 200 μM ADP, 10 μg rotenone and 50 μM $^{45}CaCl_2$ (sp. act. 500 cpm/nmol), pH 7.3. After 20 sec, an aliquot was withdrawn, filtered

through a 0.45 μm pore size filter and thoroughly washed with 0.1 M KCl. Radioactivity was measured in a scintillation counter. Line fittings were carried out by using the data analysis and technical graph program, Microcal™ Origin™, from Microcal Software, Inc. (Northampton, MA).

Binding of $^{103}Ru_{360}$ to deglycosilated mitoplasts

$^{103}Ru_{360}$ was obtained as a product of the $^{103}RuCl_3$ reaction with ammonium hydroxide as described [21]. The inhibitor was purified from other formed complexes by cation exchange chromatography. As expected, the radioactive complex exhibited a single peak at 360 nm in ammonium formiate.

Binding experiments were carried out in isolated mitoplasts and in denatured proteins proteins. Control or deglycosilated mitoplasts (0.1 mg) were incubated in 0.1 mM KCl, 0.1 mM $MgCl_2$ and 60 mM NaCl, 50 mM TRIS buffer containing the indicated concentration of the radioactive inhibitor at pH 7.3. After 10 min, an aliquot was withdrawn to measure total binding and 10 μM unlabeled inhibitor was added to remove unspecific $^{103}Ru_{360}$ binding. One minute later, mitoplasts were centrifuged at 15,000× g for 10 min. The pellet was suspended in the same buffer and used for radioactivity measurement in a gamma counter. Unspecific binding was determined by subtracting the specific binding from the total binding.

Results

Purification of mitochondria

Purified mitochondria were obtained from a crude mitochondrial pellet; the separation of these organelles from other cellular constituents with similar buoyant densities was achieved by using a Percoll density gradient. This procedure led to the removal of most contaminants of organelles containing glycoproteins, i.e. plasma membrane and endoplasmic reticulum (Table 1). The residual activity of marker enzymes in the selected purified mitochondrial population (Percoll 40%) was 12% for alkaline phosphatase and 23% for glucose 6 phosphatase. Correlatively, cytochrome oxidase specific activity (mitochondrial enzyme marker) was enriched 3.3 times in comparison with crude mitochondria. Lysosomal and peroxisomal membranes remained as contaminants in our preparations. In this respect, it has been reported that some sialic-acid containing proteins are components of lysosomal membranes [22]. Indeed, the detection of sialic-acid glycoconjugates involved oxidating conditions different from the methodology used in our experiments. We also discarded the adsorption of peroxisomal or red cell-derived catalase to our mitochondrial preparations. Beside this, the presence of catalase in rat heart mitochondria has been reported [23].

Immunodetection of glyosilated proteins in mitoplasts

To determine the presence of glycoproteins in mitoplasts, putative sugar hydroxyl groups were oxidated and DIG-labeled. The label was immunodetected with anti-DIG coupled to AP.

Western blot analysis revealed the presence of DIG-labeled proteins. A positive signal was detected in proteins of 18 kDa, 42 kDa, 81 kDa and higher molecular weight proteins ranging between 100 and 200 kDa. Under deglycosilation conditions, almost all proteins lost the DIG recognition site (Fig. 1). The signals that persist after deglicosylation could represent non-N-linked sugars.

Effect of ruthenium red and Ru_{360} on calcium uptake in deglycosilated mitoplasts

For many years, Ru_{360} was considered a contaminant of commercial ruthenium red preparations. Until recently, both species were identified as different, but structurally related compounds. Since ruthenium red was widely used to stain sugar-linked structures, a close relationship between this compound and proteoglycans was established many years ago. However, there are no reports that Ru_{360}, the most potent inhibitor of mitochondrial calcium uptake, recognizes glycopeptides or related structures. To explore if Ru_{360} inhibitory properties are related to putative glycosidic residues

Table 1. Marker enzyme activities of organelle fractions

	Acid Phosphatase (μmol/mg/min)	Alkaline Phosphatase (μmol/mg/min)	Catalase (μmol/mg/min)	Glucose-6-P Phosphatase (μmol/mg/min)	Cytochrome oxidase (ngAtO/mg/min)
Percoll 40%	3.34 ± 2.7	4.59 ± 3.3	0.035 ± 0.022	5.90 ± 3.7	1,285 ± 245
Percoll 25%	1.26 ± 0.79	34.75 ± 6.65	0.015 ± 0.009	18.97 ± 8.4	467.8 ± 97
Crude mitochondria	3.22 ± 1.9	37.08 ± 6.7	0.023 ± 0.006	24.70 ± 5.9	393.9 ± 89
Homogenate	2.57 ± 0.98	16.99 ± 7.7	0.031 ± 0.019	9.29 ± 3.2	175.75 ± 56

Note. Data represent the mean of eight different experiments ±S.D.

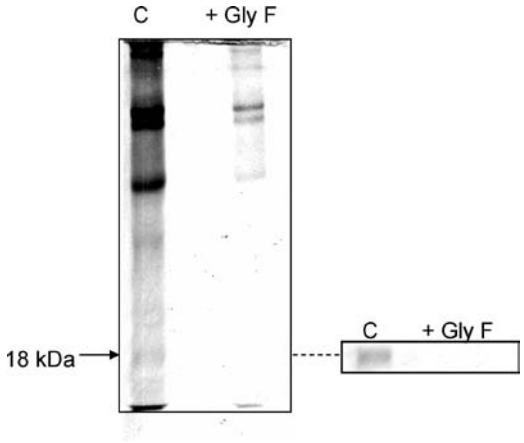

Fig. 1. Immunodetection of glycoproteins with anti-DIG-AP. Control and deglycosylated mitoplasts from purified mitochondria were labeled with DIG. 30 μg of each sample were electrophoresed and blotted onto nitrocellulose sheets. The filters were washed and incubated with anti-digoxigenin-AP and revealed with an oxydizable substrate for the enzyme. The insert is an overexposed image of the same membrane showing the 18 kDa protein in both conditions. The results shown are from one experiment that was representative of three independent replicates.

in the calcium uniporter, we examined the effect of ruthenium red and Ru_{360} on calcium uptake in mitoplasts incubated with ^{N}glycosidase F. Since deglycosilation conditions could be harmful to mitoplast integrity and to assess the role of glycosidic residues on the transport process, calcium uptake was measured in N-glycosidase F-treated mitoplasts. Uptake was also measured in the presence of CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone) to dissipate the proton gradient that maintains the mitochondrial calcium uptake. By this mean we can be sure that the calcium uniporter activity is the one observed (Fig. 2a). The Ca^{2+} influx pathway in mitoplasts was not modified after deglycosilation and as expected, CCCP avoided calcium accumulation by dissipating the inner membrane potential. Calcium uptake inhibition by Ru_{360} in control and deglycosilated mitoplasts is shown in Fig. 2b. Increasing concentrations of Ru_{360} progressively inhibited Ca^{2+} uptake in control mitoplasts. The IC_{50} of the control rate was 4 nM, in accordance with recent reports of the Ru_{360} half–maximal inhibitory concentration (2 nM) in a mitoplast inwardly rectifying current related to the mitochondrial Ca^{2+} uniporter [12], in contrast this Ru_{360} concentration, only exerts a 15% inhibition in deglycosilated mitoplasts. There was also a shift of the IC_{50} and a decrease in the maximum inhibition of calcium uptake in deglycosilated mitoplasts by ruthenium red (Fig. 2c). These data suggest that glycosidic groups located at the mitochondrial uniporter favored the interaction with ruthenium amine complexes leading to the inhibition of calcium uptake. To discard any influence of the outer membrane on the action of ruthenium complexes, we also measured Ru_{360} and ruthenium red inhibition in intact mitochondria.

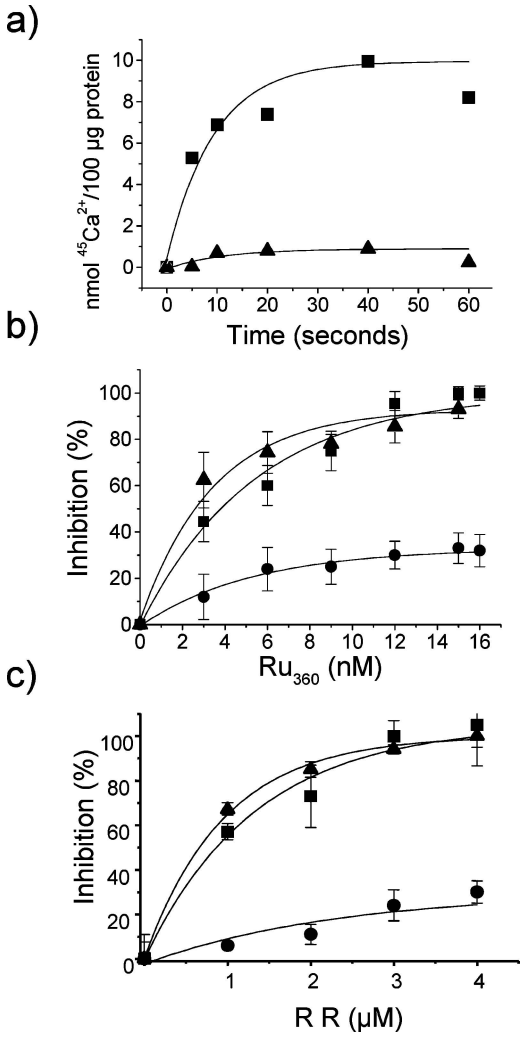

Fig. 2. Calcium uptake in mitoplasts subjected to deglycosilation. Effect of ruthenium red and Ru_{360}. (a) Mitoplasts were treated for deglycosilation as described in the Experimental Procedures section and assayed for calcium uptake by using $^{45}CaCl_2$. Control mitoplasts were incubated under the same conditions without N-glycosidase F. Calcium uptake in control and deglycosilated mitoplasts (■); Calcium uptake in control and deglycosilated mitoplasts in presence of 10 μM CCCP (▲). (b) Calcium uptake measured in deglycosilated mitoplasts in the presence of varying ruthenium red concentrations: The reaction was initiated with 100 μg of mitoplast protein and incubated for 20 sec. Deglycosilated mitoplasts (●); control mitoplasts (■); control mitochondria (▲). (c) The same experiment was carried out with Ru_{360}. Deglycosilated mitoplasts (●); control mitoplasts (■); control mitochondria (▲). Data represents the mean of three different experiments ±S.D. Temperature 25 °C; final volume 0.5 ml; pH 7.3.

The efficiency of both compounds was the same as in mitoplasts (Figs. 2b and 2c).

The relevance of glycosidic residues on calcium transport was confirmed, carrying out experiments of similar design, under conditions that led to an increased probability of the permeability transition pore opening. It is well known that

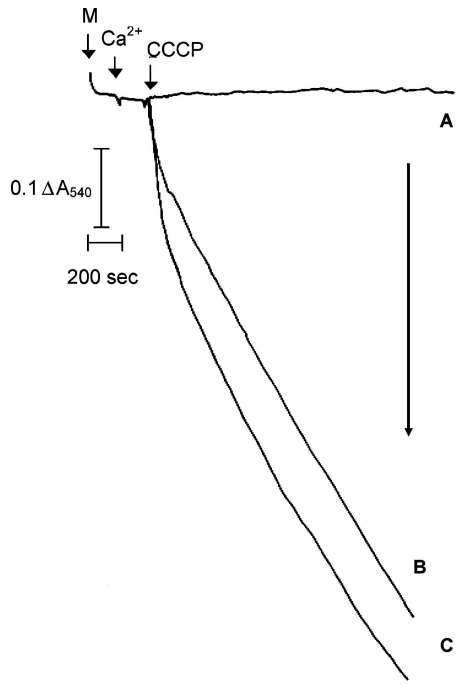

Fig. 3. Mitochondrial swelling induced by Ca^{2+} and CCCP in deglycosilated mitochondria. The incubation medium contained 250 mM sucrose, 10 mM HEPES, 10 mM succinate, 2 mM Pi, pH 7.3. Where indicated 50 μM $CaCl_2$ and 1 μM CCCP were added. 1 mg of control mitochondria (Trace A and Trace C) and 1 mg of deglycosilated mitochondria (Trace B) were used. Only traces A and B were supplemented with 75 nM of Ru_{360}. The arrow indicates increase in mitochondrial swelling. Final volume, 3 ml and temperature, 25 °C.

isolated mitochondria can undergo a sudden Ca^{2+}-dependent increase of permeability of the inner membrane, enhanced by agents that cause membrane depolarization, as is the case of uncouplers [24].

In the experiment illustrated in Fig. 3, mitochondria energized with succinate were incubated in a sucrose-based medium, and the mitochondrial volume was followed as light scattering at 540 nm, as an index of the permeability transition pore opening. Addition of 50 μM $CaCl_2$, was unable to induce pore opening *per se*, only in the presence of the uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), the mitochondrial permeability transition was induced. (Trace C). To prevent calcium redistribution through the calcium uniporter, Ru_{360} was added before calcium accumulation. Despite full de-energization at high uncoupler concentration, the mitochondrial population does not undergo permeability pore opening (Trace A). The same experiment was carried out using deglycosilated mitochondria. The final light scattering was similar than that observed in control mitochondria without Ru_{360} (Trace B), indicating the absolute requirement of Ca^{2+} accumulation for the opening of the permeability transition pore. It was also demonstrated, that deglycosilated mitochondria lost the inhibitor binding site that prevents for calcium uptake.

Interactions of Glycosidase F with the mitochondrial uniporter

It was previously described by our group and others [21, 25, 26] that Ru_{360} exerts a non-competitive inhibition on calcium uniporter activity. Calcium uptake experiments in deglycosilated mitoplasts (Fig. 2a) suggested that glycosidic residues associated with Ru_{360} and ruthenium red binding do not modify calcium binding sites. We therefore examined the inhibitory effect of lanthanum on calcium uptake in deglycosilated mitoplasts. Lanthanum exerts its function by competing with calcium for the binding and transport sites in the uniporter. As expected, deglycosilation did not have any effect on the inhibitory properties of lanthanum on calcium uptake (not shown), confirming the difference in binding sites of these inhibitors on the mitochondrial calcium uniporter.

Concanavalin A interaction with the calcium uniporter

The specific interaction of Concanavalin A with complex carbohydrate- containing cellular constituents was used to determine the type of glycosidic residues related to the mitochondrial calcium uniporter. As observed in Fig. 4, mitoplasts treated with glycosidase F are insensitive to Concanavalin A (ConA) inhibition. No mitochondrial aggregation occurred at the evaluated ConA concentrations and high respiratory rates

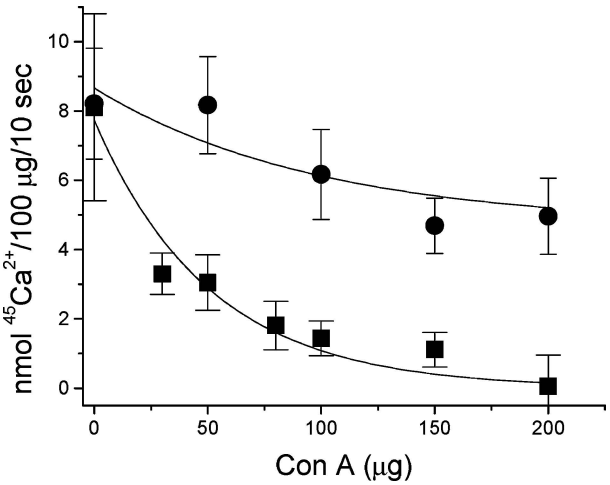

Fig. 4. Inhibition of calcium uptake by concanavalin A in mitoplasts. 0.1 mg mitoplasts were incubated under the same conditions as in Fig. 2, in the presence of different amounts of concanavalin A. Calcium uptake was measured at 10 sec. Deglycosilated mitoplasts (●); control mitoplasts (■). Data represents the mean of three different experiments ± S.D. Temperature 25 °C; final volume 0.5 m′; pH 7.3.

were observed in control and deglycosilated mitochondria, e.g. 63 ± 8 ngAtO/min/mg protein vs 68 ± 14 ng AtO/min at the higher ConA concentration. Like Ru_{360}, ConA also failed to inhibit calcium uptake after deglycosilation, suggesting a similar recognizing site for both compounds in the mitochondrial calcium uniporter.

$^{103}Ru_{360}$ binding to a deglycosilated calcium-uniporter activity fraction

To reinforce the idea that glycosidic residues are structural components of the mitochondrial calcium uniporter and to get an insight about the identity of this transporter, we analyzed $^{103}Ru_{360}$ binding to proteins contained in a semipurified mitochondrial preparation, which accumulated calcium in a reconstituted liposome system, as previously reported by our own group [15]. A calcium uniporter-like activity fraction was labeled with 100 nmol of $^{103}Ru_{360}$/50 μg protein and subjected to deglycosilation under the conditions described. The proteins were separated by denaturing electrophoresis and the polyacrylamide matrix was cut into 2 mm slices for radioactivity determinations. A representative experiment of the radioactive pattern of the excised gel is shown in Fig. 5. According to the Coumassie protein stain image, the radioactive label was associated with a 18 kDa protein and deglycosilation totally abolished $^{103}Ru_{360}$ binding to this entity. Binding of Ru_{360} to rat kidney mitoplasts was also measured using radioactively labeled Ru_{360} as described in the proper Section. In three separate mitochondrial preparations, we determined the binding of $^{103}Ru_{360}$ binding, which decrease from 1.06 ± 0.02 pmol/100 μg protein in control mitoplasts to 0.058 ± 0.005 pmol/100 μg protein after deglycosilation at 10 nM $^{103}Ru_{360.}$ This concentration is consistent with the reported high-affinity specific binding constant of this compound for the calcium uniporter [15, 21, 25].

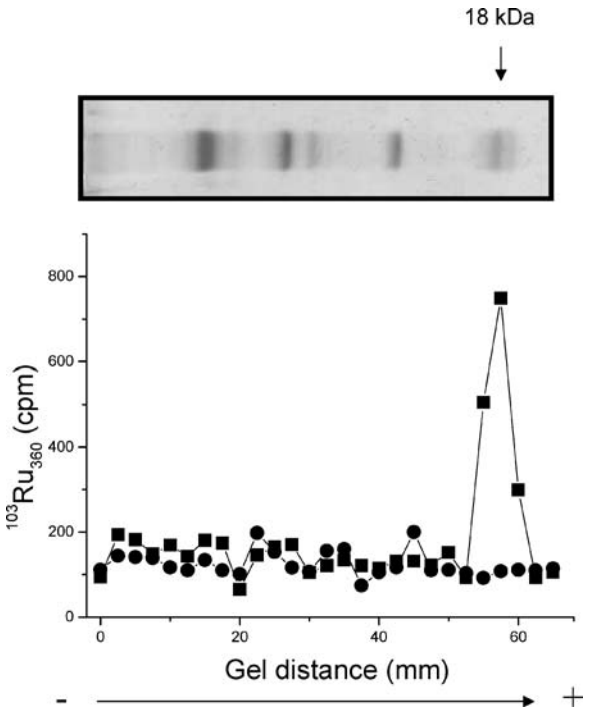

Fig. 5. Radioactivity pattern of the excised gel under control and deglycosilated conditions. Inner membrane proteins (100 μg) were treated with ^{N}glycosidase-F under the conditions described in Methods. 25 μg of control and deglycosilated proteins were subjected to denaturing electrophoresis. The gel was incubated in a humid chamber for 16 h in presence of 200 nM $^{103}Ru_{360}$ (specific activity 50 cpm/pmol) and then washed extensively with a solution containing an excess of cold Ru_{360}. The gel was cut into 2 mm slices and measured for radioactivity. Deglycosilated inner membrane proteins (●); control inner membrane proteins (■). A Coumassie stain image of the deglycosilated sample is also shown. The results shown are representative of two independent experiments.

Discussion

Glycoproteic molecules have been reported to be located in nucleus, cytoplasm and mitochondria [27]. Particularly, by metabolic radiolabeling and immunological approaches, it has been demonstrated that a 45-kDa glycoprotein with N-linked poly-mannose is a component of the inner mitochondrial membrane and is associated with at least two mitochondrial enzyme complexes [28]. Accordingly, the group of Gasnier [29], reported that mitochondrial membranes contain several specific glycosyltransferases involved in the maturation of glycoproteins. In 1994, Mironova's group reported a 40-kDa glycoprotein and a smaller peptide that exhibited ruthenium-red sensitive Ca^{2+}-channel properties when reconstituted into planar phospholipid bilayers., although the glycoprotein was later identified as a plasma contaminant with high homology to human plasma orosomucoid [7].

A relationship between ruthenium red and glycoproteins associated with the mitochondrial calcium uniporter is not new, and arose from a supposed specificity of this dye for proteoglycans. However, there are no reports that Ru_{360} recognizes glycopeptides or related structures. Importantly, this compound has been shown to exclusively block the mitochondrial calcium uniporter in isolated cardiac myocytes [25].

Our results show the presence of N-glycosilated proteins associated with the calcium uniporter, presumably localized at the cytosolic side of the mitoplast membrane, that could function as a recognition site for the ruthenium polycationic inhibitors of calcium uptake. The function of N-linked oligosaccharides of some proteins has been studied. Keutmann *et al*. [30], reported that a conformational change, induced by deglycosilation in human chorionic gonadotropin, led to a lower hormonal activity. On the other hand, desialylated human erythropoietin was more active

than the sialylated hormone, as measured through a bioassay system *in vitro* [31].

As Ruthenium red and Ru_{360} are non-competitive inhibitors of the mitochondrial calcium uniporter [10, 12], the calcium conducting channel would no be expected to contain the glycosidic residues, as we confirmed in the experiments using the competitive inhibitor lanthanum. Since, apparently, oligosaccharides do not contribute to the active conformation of the uniporter, we propose that the sugar chains belong to an accessory molecule, that interacts with the calcium uniporter exerting a regulatory role. In this respect, it has been suggested that the uniporter could be constituted by at least two subunits. Igvavboa and Pfeiffer [32] proposed that the regulation of the uniporter could be a response to association-dissociation cycles of this factor, when calcium binds to the activation site. One of those subunits could be a dissociable factor related to glycoproteins.

Our observation that Concanavalin A (ConA) inhibited calcium uptake suggested that the nature of the glycosidic residues involved in the ruthenium polycationic inhibitor recognition, could be α-mannosyl and/or α-glucosyl residues. Relevant to this speculation is the report of the behavior of various oligosaccharides and glycopeptides in a ConA-Sepharose column. It was concluded that the presence of two binding residues linked to the trimannosyl core (structure common to all N-linked sugar chains) is required for a sugar chain to be retained on the column. A complex-type sugar with three outer binding chains passes through the column without interaction [33].

The present study provides evidence that glycosidic groups located at the mitochondrial uniporter favored the interaction with ruthenium amine complexes. The nature of such interaction had not yet been explored, however, chemical studies demonstrated that in an aqueous environment, both ruthenium red and Ru_{360} are polyvalent cations [21, 34]. As Ru_{360} has the linear backbone N-Ru-O-Ru-N, in which each ruthenium atom is positively charged with a remaining charge delocalized between the Ru-O-Ru [21], it would be expected to be very responsive to negative charges in proteins, lipid membranes and cation carriers. In a previous report, our group demonstrate that ruthenium red and Ru_{360} are mutually exclusive and do not compete for the high affinity Ca^{2+}-binding sites with lanthanum [26]. A mutual exclusivity indicates that both compounds act on the same specific site in the uniporter. It is plausible, therefore, that the findings observed in the inhibition behavior of both compounds under deglycosilated conditions, arise from the elimination of anchoring reactive groups, i.e. aldehyde or carboxyl groups belonging to the carrier or to a recognition site represented by an accessory protein.

In the past years we reported the identification of a mitochondrial protein (18 kDa), which binds Ru_{360} with high affinity [15]. This protein was susceptible to N-deglycosilation as demonstrated in the experiments of radiolabeling and molecular weight separation after deglycosilation (Fig. 5). This protein would be an attractive candidate for possessing the glycosidic residues that interact with the ruthenium polycationic inhibitors of the calcium uniporter.

Acknowledgments

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