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

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Received June 7, 2004; accepted June 20, 2004

The mitochondrial calcium uniporter behaves as a cooperative mechanism, where the velocity is dependent on \([\text{Ca}^{2+}]_{\text{ex}}\). 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 polycationic ruthenium compounds. In this study, calcium uptake activity was reconstituted into cytochrome oxidase vesicles by incorporating solubilized mitochondrial proteins. Calcium accumulation plotted against increasing \(\text{Ca}^{2+}\) concentrations followed a sigmoidal behavior with a Hill coefficient of 1.53. The uptake was sensitive to ruthenium polycationic inhibitors, e.g. ruthenium red and Ru360. 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 Ru360, while the second activity was insensitive to Ru360 and was associated with proteins focused at very acidic isoelectric points. By using a thiol-group crosslinker and radiolabeled Ru360, 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 \(\text{Ca}^{2+}\) and \(\text{Pr}^{3+}\) 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 Ru360 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.

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Key to abbreviations: CCCP, Carbonyl-cyanide m-chlorophenyl-hydrazone; EGTA, Ethylene glycol-bis (2-aminoethyl)ether-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 Bystolyte 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 H3PO4-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 KH2PO4, pH 7.0 to eliminate ampholytes, except for refocusing experiments. Each fraction (60–90 µg) was incubated with 5 µL of the labeled 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

\[ \frac{v}{V_{\max}} = \frac{[S]}{K_s + [S]} \]

where \( v \) is the rate of substrate uptake, \( V_{\max} \) is the maximum rate, \( [S] \) is the substrate concentration, and \( K_s \) is the dissociation constant.