Journal of Bioenergetics and Biomembranes, Vol. 26, No. 2, 1994

Purification of the Channel Component of the Mitochondrial Calcium Uniporter and Its Reconstitution into Planar Lipid Bilayers

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Received July 14, 1993; accepted November 2, 1993

The purification of the channel-forming component of the mitochondrial calcium uniporter and its channel properties are described. After ethanol and 50% ethanol–water extraction of mitochondria from beef heart or perfused rat liver, the extract was passed through thiopropyl-Sepharose 6B column, and absorbed components were eluted with 2-mercaptoethanol, followed by gel-filtration on Sephadex G-15. The last fraction eluted (M_r about 2000) was then subjected to reverse-phase high-performance liquid chromatography. Of the more than 10 distinct peaks, only one showed specific Ca^{2+}-channel activity in BLM with properties similar to earlier, less extensively purified preparations, i.e., conductance of 20 pS and multiples thereof, clustering of channels, participation of 2 or more subunits in channel formation, and sensitivity to 1 μM ruthenium red. Voltage sensitivity and cooperativity between channels are described. The Ca^{2+}-binding glycoprotein with which the peptide was associated was found to have high homology with human acid α_1-glycoprotein (orosomucoid) and to show identity with beef plasma orosomucoid in the Ouchterlony immunodiffusion test.

KEY WORDS: Acid α_1-glycoprotein; black-lipid membrane; calcium uniporter; channel; orosomucoid; reconstitution.

INTRODUCTION

The mitochondrial calcium uniporter is a transport system that is responsible for the membrane potential-driven, ruthenium red-sensitive uptake of Ca^{2+} and some other divalent cations (Sr^{2+}, Mn^{2+}, Ba^{2+}) into the matrix space; see review by Gunter and Pfeiffer (1990). Mironova *et al.* (1982) have obtained a preparation from beef heart mitochondria containing a 40-kDa glycoprotein and a smaller peptide that exhibited ruthenium red-sensitive Ca^{2+}-channel properties when reconstituted into planar phospholipid bilayers (BLM).[5] The Ca^{2+}-transporting glycoprotein was reported to be a complex of the glycoprotein part and a small channel-forming component that may be dissociated from it (Mironova and Utesheva, 1989). Since the channel activity was lost on treatment with protease, it probably is a peptide, but other evidence in favor of this is not yet available. We have recently found that antibodies raised against this glycoprotein–peptide complex inhibit the calcium uniporter in rat liver mitoplasts and that the antigen(s) are specifically localized in mitochondria in fibroblasts (Saris *et al.*, 1993). We now report the purification of the channel-forming component—here called

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[5] Abbreviations: BLM, black-lipid membranes, planar lipid bilayers; Tris, Tris(hydroxymethyl)aminomethane.

0145-479X/94/0400-0231$07.00/0

peptide—to homogeneity and describe its channel-forming properties when reconstituted into a planar lipid bilayer. We have partially sequenced the glycoprotein-containing fraction, and these sequences correspond to those of plasma acid α_1-glycoprotein, also called orosomucoid.

MATERIAL AND METHODS

Rat livers were perfused with physiological salt solution prior to the preparation of mitochondria by a conventional procedure and mitoplasts by digitonin treatment of mitochondria (Schnaitman and Greenawalt, 1968). Mitochondrial protein was estimated by a biuret procedure in the presence of cholate using bovine serum albumin as standard. Extraction and purification of the channel protein is described in the Results section. Beef plasma orosomucoid was isolated as described by Bystrova and Belenky (1985). Antisera against the mitochondrial protein fraction and against beef plasma orosomucoid were raised in rabbits, the former as described by Saris *et al.* (1993), the latter by primary multiple intracutaneous injections of 270 μg protein in 500 μl complete Freund's adjuvant, with three booster injections at 4–5 week intervals. The IgG fractions of the antisera were purified and immunofixation carried out as described by Saris *et al.* (1993).

Ca^{2+} transport was followed with the radioisotope method (^{45}Ca was obtained from The Radiochemical Centre, Amersham, U.K.), counting the radioactivity of mitochondria retained on 0.45 μm pore-size filters (Saris and Allshire, 1989). The content of divalent cations of the reagents was reduced by passing through a CHELEX column.

The channel activity was measured using 4-nm-thick planar bilayers formed from lipid monolayers raised past an aperture between two compartments according to Montal (1974). The cuvette contained 3 ml 20 mM Tris-HCl buffer, pH 7.4, both in the *cis* and *trans* compartments. Ag-AgCl electrodes for measuring *trans*-membrane current were connected to the electrolyte solutions in the *cis* and *trans* compartments via agarose bridges. The electrode for measuring potential was placed in the *cis* compartment. Proteins and salts were introduced as aqueous solutions. Lipids were extracted from bovine brain as described by Hara and Radin (1978), dissolved in hexane (3 mg/ml), and allowed to form the monolayers that create a bilayer on raising the level in the compartments past the aperture. The *trans*-membrane current was recorded with the aid of an operational amplifier (OPA 101, Burr-Brown Corp., Tucson, AZ) (Niles *et al.*, 1988) connected to a Tectronix 2211 Digital Storage oscilloscope (Niles *et al.*, 1988). The membrane voltage was clamped. The potential of the *trans*-compartment was taken as zero. The ion-selective properties of incorporated proteins were determined by measuring the potential of zero current in the presence of different salt concentration. A twofold ion gradient was used. The conductance of the membrane in the absence of protein was about 3–10 pS/mm^2.

Automated amino acid sequence analysis was performed on an Applied Biosystems 477A/120A pulsed-liquid phase sequencer (Applied Biosystems, Foster City, California 94403-9991, USA). Released PTH-amino acids were identified on-line at 269 nm by reverse-phase HPLC.

RESULTS

Extraction of the Channel-Forming Peptide

Mitochondria from perfused rat liver, 20 mg protein/ml, were extracted in 94% (w/v) ethanol (−20°C) at pH 7.0 at 4°C for 20 min under constant stirring. The final concentration of ethanol was 88%. After centrifugation (5000 *g*, 15 min, 4°C), the supernatant was discarded and the pellet extracted with 50% ethanol (−20°C) for 30 min at 4°C with stirring, followed by centrifugation as above. The supernatant from 1 g mitochondria was combined (the pH was brought to 8.0 with ammonia), evaporated in vacuum to 10 ml, and centrifugated at 45,000 *g* at 4°C for 1 h.

Purification of the Channel-Forming Peptide

Step 1. Thiopropyl-Sepharose 6B Column Chromatography. The extract from 1 g mitochondria was applied on the thiopropyl-Sepharose 6B column, volume 2 ml, at a rate of 5 ml/h. The column was washed with 10 ml of 20 mM ammonium formate buffer, pH 8.0, at the same rate. The bound components were eluted with the same buffer containing 50 mM 2-mercaptoethanol at the rate of 8 ml/h. The eluate was evaporated to 2 ml.

Step 2. Sephadex G-15 Gel Filtration. The eluate was applied on the Sephadex G-15 column (80 ml). A typical elution pattern is presented in Fig. 1. The

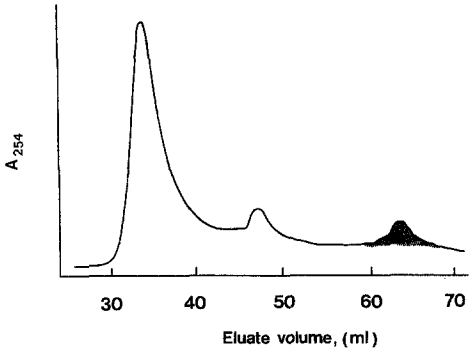

Fig. 1. Typical elution profile in gel filtration on Sephadex G-15 of mitochondrial extract. Channel-forming activity was found in the third, small-molecular weight fraction (hatched area).

channel activity was found only in the third fraction (Fig. 1, hatched area). It is of interest that when the unbound fraction in Step 1 was subjected to gel filtration, no fraction had any channel activity.

Step 3. HPLC Chromatography. The channel-forming fraction was applied on a reverse-phase microbore column (Vydac C-18, 218-TP52, Hesperia, 92345 USA) coupled to a Beckman System Gold high-performance liquid chromatograph. Fractions were released by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water (0–60% acetonitrile in 30 minutes); see Fig. 2. The flow rate was 0.2 ml/min.

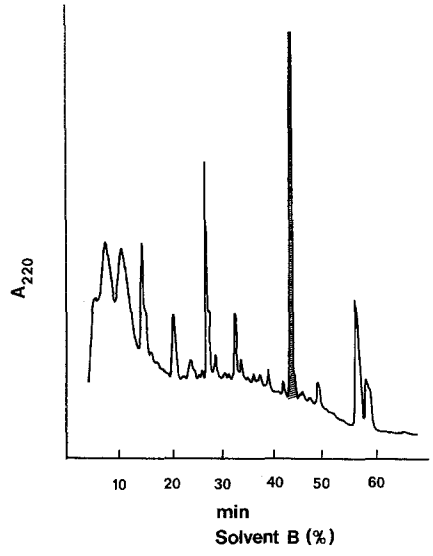

Fig. 2. Elution pattern in HPLC fractionation of the active fraction from the gel filtration (Fig. 1). A gradient elution with solvent A, 0.1% trifluoroacetic acid, and increasing proportions of solvent B, acetonitrile. The channel-forming activity was found in the fraction (hatched area) eluted with 42–44% acetonitrile.

Fig. 3. Channel activity in BLM reconstituted with the fraction in Fig. 2, hatched area. The chamber solution contained 20 mM Tris-HCl buffer, pH 7.4, and 10 mM $CaCl_2$. The preparation, 5 μg/ml, was introduced in chambers to both sides of the membrane. A voltage of 100 mV was applied to the membrane.

Ca^{2+}-Channel Properties of the Peptide

The active fractions obtained after Steps 2 and 3 in the purification scheme increased the membrane conductance only when added to both compartments separated by the membrane. No channels were formed when the protein was added only to one side of the membrane. Since conductance was studied in the presence of Ca^{2+}, it is not known whether the reconstitution requires Ca^{2+}. The experiment was done also with small concentrations of the active fractions in Figs. 1 and 2 added to compartments on both membrane sides. Then the *trans*-membrane current was changed sharply between discrete values. This fact indicates the formation of ionic channels in the bimolecular lipid membrane (BLM); see Figs. 3–5. The minimum conductance was 10 pS (in the presence of 10 mM $CaCl_2$) and frequently channels with conductances 20 and 40 pS were observed (Fig. 3). When more channels were active, there was evidence of stepwise changes in conductance, more than 40 pS but multiples of 10 pS (Fig. 5). This may indicate that the peptide channels form interacting clusters.

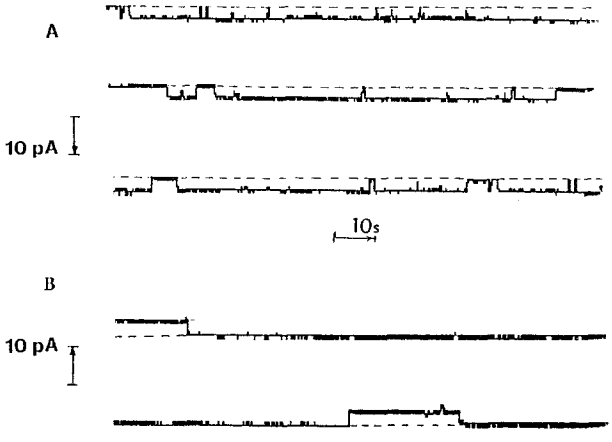

Fig. 4. Dependence of single-channel activity in BLM reconstituted with the active fraction shown in Fig. 2 versus membrane voltage. In A, the applied voltage was 100 mV; in B, 100 mV. The other experimental conditions were as in Fig. 3.

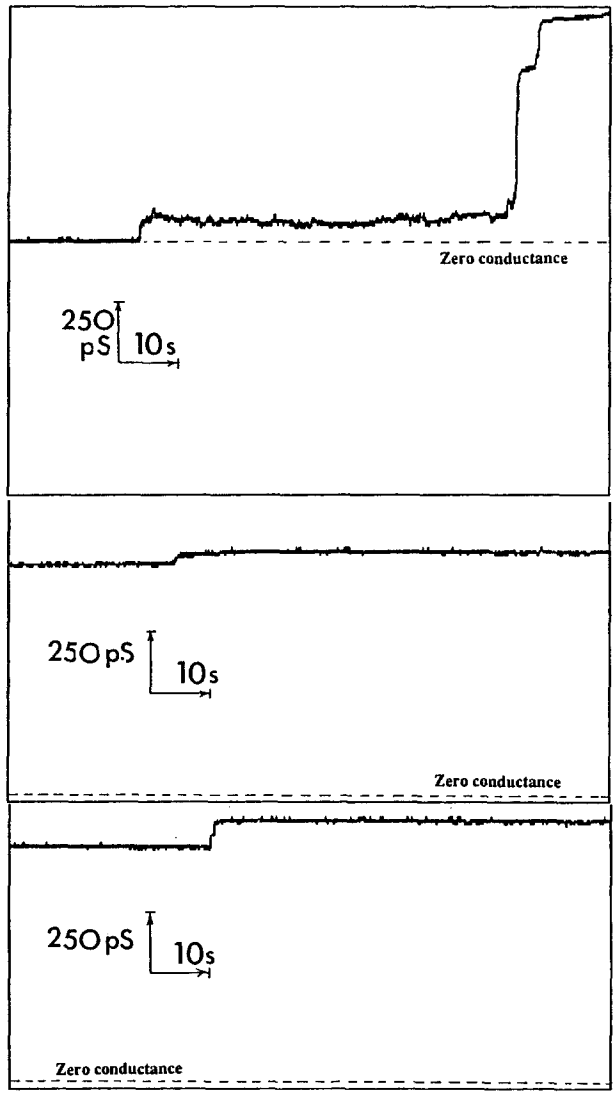

Fig. 5. Dependence of conductance on the concentration of the active chanel component in Fig. 1. The experimental conditions were as in Fig. 3.

Figure 4 shows currents at different membrane potentials. It is seen that single channel activity is dependent on the membrane potential. At positive potential there was an increased probability for the channel being in the open state (Fig. 4A). At negative potential the channel is closed (Fig. 4B).

The conductance/$[Ca^{2+}]$ relationship is shown in Fig. 6 using the active fraction after Step 2 purification. The relationship is linear over the concentration range 0.1–15 mM.

The single channel and the cluster of channels are strongly selective for Ca^{2+} (Fig. 7). The potential obtained at a 2-fold gradient of Ca^{2+} in the presence

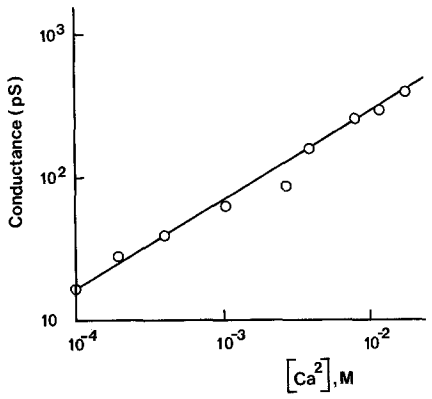

Fig. 6. Dependence of BLM conductance on the concentration of Ca^{2+}. The experimental conditions correspond to those in Fig. 3.

of the peptide was 8 mV, which is close to the Nernst potential for a bivalent cation. It follows then that the channels do not possess Cl^- conductance. Nor are K^+ or Na^+ ions transported through these channels, which was earlier found to hold for the glycoprotein–peptide complex too (Mironova *et al.*, 1982).

The conductance was found to be sigmoidally dependent on the concentration of the channel component (Fig. 8). This suggests that more than one peptide molecule participates in forming the channel, which was also observed earlier for the glycoprotein–peptide complex (Mironova *et al.*, 1982).

The specific inhibitor of the mitochondrial calcium uniporter, ruthenium red, inhibited both

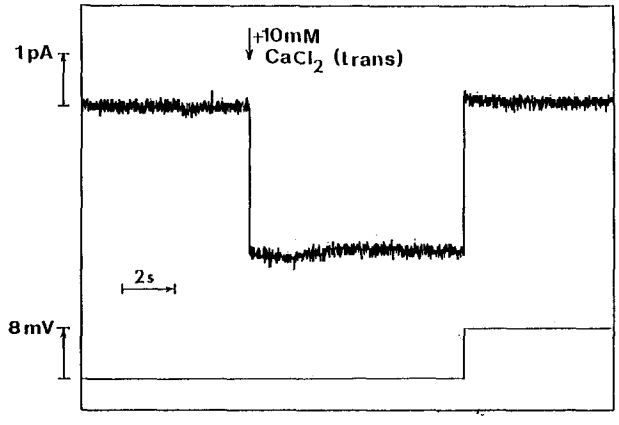

Fig. 7. Selectivity of the Ca^{2+} channel. Initially, 10 mM $CaCl_2$ was present in both compartments. The arrow indicates the moment when additional $CaCl_2$ was added to the *trans*-compartment up to the final concentration of 20 mM. After that, the current was compensated by applying the external voltage at the point indicated by a step in the voltage trace. The other experimental conditions were as in Fig. 3.

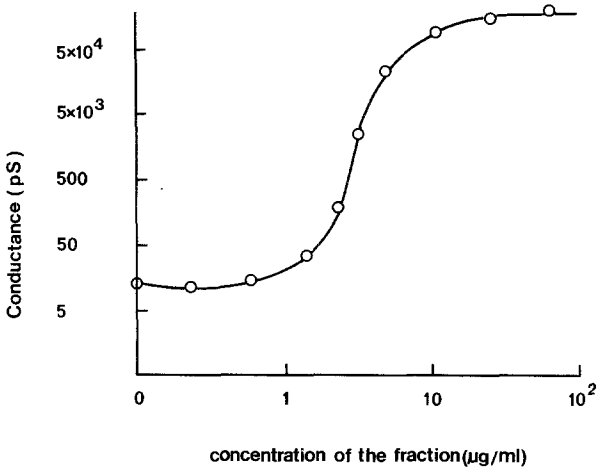

Fig. 8. Dependence of BLM conductance on the concentration of channel-forming peptide. The experimental conditions correspond to those in Fig. 3. The peptide from fraction 3 in Fig. 1 was used.

single channel activity at 1 μM concentration (Fig. 9), as well as the activity of clusters of channels (Fig. 10). Ruthenium red similarly inhibited the channel activity of the glycoprotein-peptide complex (Fig. 11). The clusters of channels were progressively inhibited by 4 μM ruthenium red. It is noteworthy that opening and closing of channels rarely occurred in a stepwise fashion but most of the time was an all-or-nothing event. This shows strong cooperativity between channels in the clusters.

Comparison of the Ca^{2+}-Binding 40-kDa Glycoprotein with Plasma Acid α_1-Glycoprotein

Amino Acid Sequences. The 40-kDa band glycoprotein present in a crude channel-forming fraction was isolated from a SDS-polyacrylamide gel both by passive diffusion (Kurt and Stoffel, 1990) and micro-preparative gel electrophoresis (Baumann and Lauraeus, 1993). The purified glycoprotein was alkylated, treated with 4-vinylpyridine (Hawke and Yuan, 1987), and digested with trypsin. Released peptides were isolated by reverse-phase microbore HPLC and subjected to automated Edman degradation as described earlier. The amino acid sequences obtained revealed a high homology with human plasma orosomucoid (Schmid *et al.*, 1973); see Fig. 12. Purified beef plasma orosomucoid and the purified glycoprotein (beef heart mitochondria fraction) were subjected to peptide map analysis and deglycosylation experiments which all indicated a close homology of these two proteins (data not shown).

Ouchterlony Immunodiffusion. Figure 13 shows an immunodiffusion analysis of the glycoprotein from beef heart mitochondria and beef plasma orosomucoid using the antiserum raised against the beef mitochondria glycoprotein–peptide complex. Immunoprecipitation lines were formed against these two proteins. These merged completely, showing that the antibodies did not discriminate between these proteins.

Antibodies against Orosomucoid Do Not Inhibit the Calcium Uniporter Nor React with Mitochondria

Figure 14 shows that IgG from antiserum raised against beef plasma orosomucoid had at most a slight inhibitory effect on the uptake of Ca^{2+} by rat liver mitoplasts (open circles) compared to the control, in which mitoplasts had been incubated with medium alone. It should be noted that IgG from nonimmunized rabbits also has a slight inhibitory effect; cf. Fig. 1 in Saris *et al.* (1993).

When antiserum raised against beef plasma orosomucoid was applied to preparations of beef heart or human fibroblasts, no immunofixation of

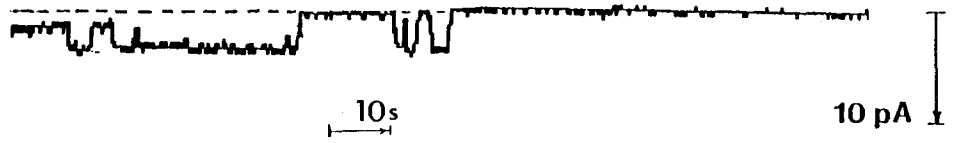

Fig. 9. Inhibition of single channel activity by ruthenium red. Experimental conditions were as in Fig. 3.

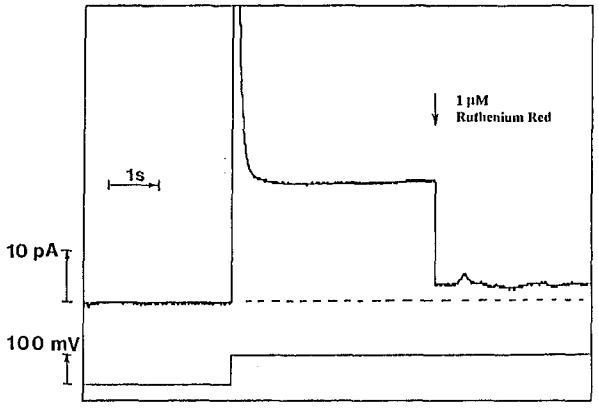

Fig. 10. Inhibition of cluster channel activity by ruthenium red. Experimental conditions were as in Fig. 5.

mitochondria nor other cellular components was found (not shown).

DISCUSSION

Our results suggest that we have purified a low-molecular weight peptide from perfused rat liver mitochondria and reconstituted it into planar lipid membranes. It formed Ca^{2+}-selective channels. The selectivity was very high in comparison with anions. The channels were strongly inhibited by ruthenium red—a potent inhibitor of the Ca^{2+} uptake in mitochondria (Moore, 1971; Reed and Bygrave, 1974). The ability of the compound to form channels was sensitive to proteinase treatment

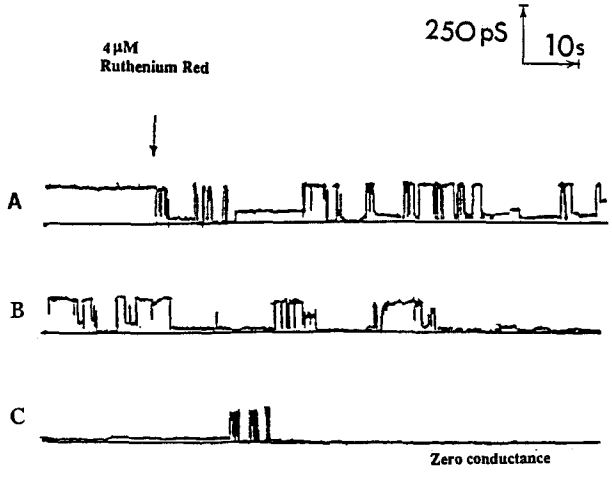

Fig. 11. Inhibition by ruthenium red of channel activity of channels formed by glycoprotein–peptide complex. Concentration of protein was 6 mg/ml. The conductivity trace A continues as B, followed by C.

(Mironova and Utesheva, 1989). Based on these properties of the compound, we believe it to be the channel-forming peptide of the mitochondrial calcium uniporter. However, in order to see channel activity with the BLM technique, relatively high concentrations of Ca^{2+}—in the millimolar range—have to be used (see Fig. 6), while mitochondria are able to accumulate Ca^{2+} from micromolar or even submicromolar concentrations. Using the proteoliposome technique, smaller concentrations of Ca^{2+} could be used. In preliminary experiments it was found that proteoliposomes reconstituted with the partially purified preparation of the glycoprotein–peptide complex from beef heart mitochondria mediated membrane-potential-driven Ca^{2+} uptake with a K_M of 20 μM that was inhibited by ruthenium red with an IC_{50} of 2–3 nM (unpublished observation of S. Zhou, G. Mironova, and K. D. Garlid, *Biophys. J.* **64**, A80, 1993). This supports our interpretation that we are dealing with the uniporter, since the channel peptide was purified from the glycoprotein–peptide complex.

For channel formation the peptide must be added to both sides of the membrane. This suggests that the channel is formed by two (or more) subunits like Gramicidin D (Urry, 1972).

Let us compare the Ca^{2+}-transporting properties of the peptide with Ca^{2+} channels isolated from mitochondria earlier. Jeng and Shamoo have isolated a small peptide—called calciphorin—with Ca^{2+}-ionophoretic properties (Jeng and Shamoo, 1980a,b). Since reconstitution of this peptide in BLM has not been reported, we cannot compare the channel-forming properties of calciphorin with those of the peptide we have reconstituted into BLM. We have earlier described the channel-forming activity of a Ca^{2+}-binding glycoprotein and shown it to be due to a low-molecular weight peptide present in it (Mironova *et al.*, 1982; Mironova and Utesheva, 1989). From gel-filtration studies using Sephadex G-10 and G-15, the M_r was estimated to be about 2000 (Pronevich *et al.*, 1982). Antibodies against this preparation inhibit the calcium uniporter at micromolar concentrations of Ca^{2+} (Saris *et al.*, 1993), which supports the conclusion that we have purified a component of the uniporter. The properties of the channel-forming peptide isolated in the present study and the channel formed by the previously studied Ca^{2+}-binding glycoprotein–peptide complex (Mironova *et al.*, 1982) are identical. Both preparations when reconstituted into BLM

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1       5       10      15      20      25      30      35      40
M A L S W V L T V L S L L P L L E A Q I P L C A N L V P V P I T N A T L D Q I T G K

W F Y I A S A F/A R N E E Y N K S V Q E I Q A A/T F F Y F T P N K T E D T I F L R E
Q F Y I X R A  A  R N X E Y X K      A I Q A  A  F F Y L T P X K

Y Q T R Q D Q C I Y N T T Y L N V Q R E N G T I S R Y E/V G G Q E H V/F A H L L I L
                                                V  E  R D X E H  F  V H L L L A

R D T K T Y M L A F D V N D E K N W G L S F/V Y A D K P E T T K E Q L G E F Y E A L
                          N V G V S  F  Y A D K P E V T Q E Q K

D C L R I P K S D V V Y T D W K K D K C E P L E K Q H E K E R K Q E E G E S
                                      K Q H E E E R K
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Fig. 12. Amino acid sequences of the 40-kDa glycoprotein from beef heart mitochondria compared to human plasmia acid α_1-glycoprotein. Upper rows, amino acid sequences of human acid α_1-glycoprotein (Schmid *et al.*, 1973) with indication of amino acid number. Lower rows, determined sequences (italics) of 40-kDa glycoprotein in beef heart mitochondrial fraction aligned with homologous sequences. *X*, no signal detected.

formed Ca^{2+}-selective, ruthenium red-sensitive channels. The channels had the same amplitudes of 20 pS or multiples thereof. The sensitivity to ruthenium red was equally high, with potent inhibition at 1 μM. In both cases more than one subunit was needed for channel formation.

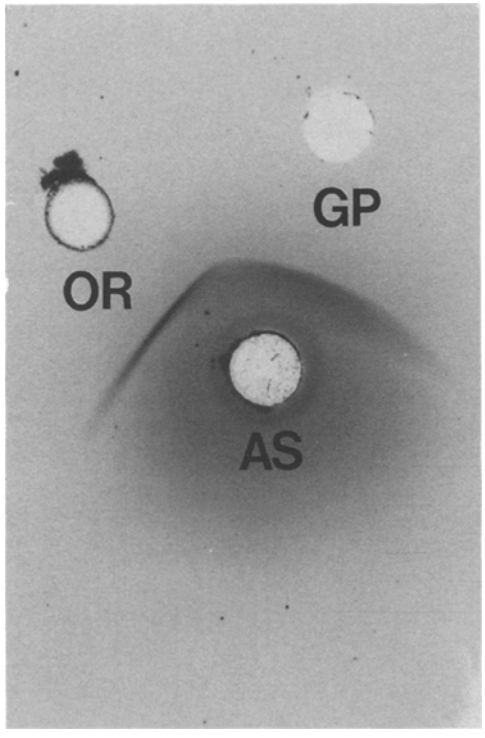

Fig. 13. Immunodiffusion test according to Ouchterlony of the reaction between IgG from acid α_1-glycoprotein antiserum (AS) and beef plasma acid α_1-glycoprotein (OR) and glycoprotein–peptide complex from beef heart mitochondria (GP), respectively.

There are some differences in the Ca^{2+}-binding affinities between the glycoprotein–peptide complex and the glycoprotein from which the peptide has been removed. It was found that the complex contained both high-affinity ($K_d = 3.8 \times 10^{-6}$ M) and low-affinity ($K_d = 4.3 \times 10^{-5}$ M) Ca^{2+}-binding sites, while the glycoprotein alone only possessed the high-affinity sites. It was therefore concluded that the peptide part contained the low-affinity sites (Utesheva *et al.*, 1989). It is of interest that it has been suggested that low-affinity Ca^{2+}-binding sites are directly involved in the transport of this cation by the uniporter (Happel and Krall, 1979).

The data reported here indicate that the 40-kDa glycoprotein probably is a contaminating plasma protein. Indeed, an Ouchterlony immunodiffusion test (Fig. 14), using polyclonal antisera raised against the glycoprotein–peptide complex, showed full identity between the glycoprotein present in the mitochondrial preparation and beef plasma orosomucoid. Its presence in association with a channel-forming peptide (Mironova and Utesheva, 1989) would then be due to copurification. This small peptide may not form immunoprecipitates and thus would not be detected in the Ouchterlony test. One may consider the possibility that the glycoprotein in the mitochondrial extract belongs to the same protein family as plasma orosomucoid. Indeed, a 52-kDa membrane-bound form of orosomucoid has been described by Gahmberg and Andersson (1978) in leukocyte plasma membranes, from which the smaller 41-kDa plasma protein could be formed. Antibodies raised against the 52-kDa glycoprotein were fixed to the plasma membranes of leukocytes

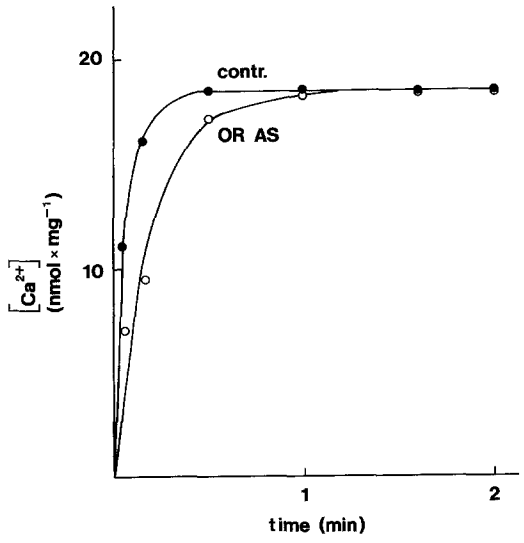

Fig. 14. Antibodies against beef plasma acid α_1-glycoprotein do not inhibit the mitochondrial calcium uniporter. Mitoplasts, 0.27 mg protein, were incubated with 0.24 mg antibody-containing IgG for 1 h at 0°C in a volume of 40 μl. For the measurement of Ca^{2+} transport by the calcium-sensitive electrode technique, the mitoplasts were then transferred to the incubation medium with a final volume of 1.25 ml at room temperature. The medium contained 200 mM sucrose, 20 mM KCl, 10 mM Hepes, pH 7.4, 1 mM KH_2PO_4, 2 mM succinate, 6 μM rotenone, and 5 μM Ca^{2+} labelled with ^{45}Ca. Aliquots of 200 μl were removed for filtration through 0.45 μm pore filters, washed with ice-cold medium, and the radioactivity of the filters counted. In the control the incubation at 0°C was done only with phosphate-buffered physiological NaCl solution. OR AS, IgG from acid α_1-glycoprotein antiserum.

but did not bind to human fibroblasts. Since the glycoprotein studied by us is a 40-kDa protein, it is not likely to contain a membrane-anchoring part and to be a genuine mitochondrial component. One cannot, however, exclude that another protein with this mass is present in the 40-kDa fraction as a minor, nondetected component.

The main result of this study is the isolation of the pure peptide that forms Ca^{2+} channels in lipid membranes. This result is a key stage to elucidate the molecular structure and mechanism of functioning of the mitochondrial calcium uniporter by methods of molecular biology and reconstitution into lipid bilayers.

ACKNOWLEDGMENTS

This study was supported by grants from the University of Helsinki, from the Sigrid Jusélius foundation to Dr. Mironova, and the Finnish Society of Sciences and Letters to Dr. Saris. The excellent technical assistance of Mrs. Kaija Niva is gratefully acknowledged. We are thankful to Dr. Jorma Ohisalo at the Department of Medical Chemistry, University of Helsinki, for raising antibodies against beef plasma orosomucoid.

REFERENCES

Baumann, M., and Lauraeus, M. (1993). *Anal. Biochem.*, in press.
Bystrova, N. K., and Belenky, D. M. (1985). *Lab. Delo,* **4**, 209–211.
Gahmberg, C. G., and Andersson, L. C. (1978). *J. Exp. Med.* **148**, 507–521.
Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **258**, C755–C786.
Happel, R. D., and Krall, A. R. (1979). *Biochem. Soc. Trans.* **7**, 1311–1312.
Hara, A., and Radin, N. (1978). *Anal. Biochem.* **90**, 420–426.
Hawke, D., and Yuan, P. (1987). *ABI User Bull.*, No. 28.
Jeng, A. Y., and Shamoo, A. D. (1980a). *J. Biol. Chem.* **255**, 6897–6903.
Jeng, A. Y., and Shamoo, A. D. (1980b). *J. Biol. Chem.* **255**, 6904–6912.
Kurt, J., and Stoffel, W. (1990). *Biol. Chem. Hoppe-Seyler* **371**, 675–685.
Mironova, G. D., and Utesheva, Zh. A. (1989). *Ukr. Biochem. J.* **61**, 48–54.
Mironova, G. D., Sirota, T. V., Pronevich, L. A., Trofimenko, N. V., Mironov, G. P., Grigorjev, P. A., and Kondrashova, M. N. (1982). *J. Bioenerg. Biomembr.* **14**, 213–225.
Montal, M. (1974). *Meth. Enzymol.* **32**, 545–554.
Moore, C. L. (1971). *Biochem. Biophys. Res. Commun.* **42**, 298–305.
Niles, W. D., Levis, R. A., and Cohen, F. S. (1988). *Biophys. J.* **33**, 327–335.
Pronevich, L. A., Mironov, G. P., and Mironova, G. D. (1982). In *Chemistry of Peptides and Proteins*, Vol. 1, Academic Press, New York, pp. 457–461.
Reed, K. C., and Bygrave, F. L. (1974). *Biochem. J.* **140**, 143–155.
Saris, N.-E. L., and Allshire, A. (1989) *Meth. Enzymol.* **174**, 68–84.
Saris, N.-E. L., Sirota, T. V., Virtanen, I., Niva, K., Penttilä, T., Dolgachova, L. P., and Mironova, G. D. (1993). *J. Bioenerg. Biomembr.* **25**, 307–312.
Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M., and Nanno, S. (1973). *Biochemistry* **12**, 2711–2724.
Schnaitman, C. A., and Greenavalt, J. W. (1968) *J. Cell Biol.* **38**, 158–175.
Urry, D. W. (1972). *Proc. Natl. Acad. Sci.* **69**, 1610–1614.
Utesheva, Zh. A., Sirota, T. V., and Mironova, G. D. (1989). *Ukr. Biochem. J.* **61**, 54–58.