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Isolation and Properties of Ca^{2+}-Transporting Glycoprotein and Peptide from Beef Heart Mitochondria

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Abstract

The 40,000-dalton glycoprotein and 2000-dalton peptide inducing selective Ca^{2+}-transport through bilayer lipid membranes were isolated from beef heart homogenate and mitochondria. Micromolar concentrations of these substances were found to increase the conductivity of membranes by 3–4 orders. Transmembrane Ca^{2+} gradient induces an electric potential difference whose magnitude is close to the theoretical for ideal Ca^{2+} selectivity. The inhibitor of mitochondrial Ca^{2+} transport, ruthenium red, abolishes both the glycoprotein- and peptide-induced Ca^{2+} transport in bilayer lipid membranes. Thiol groups essential for Ca^{2+} transport activity were revealed in the glycoprotein and peptide. Addition of these substances to rat liver mitochondria induces Ca^{2+}-dependent inhibition of the state 3 respiration that can be released by uncouplers (oligomycin-like effect).

Key Words: Mitochondria; transport; calcium; bilayer lipid membrane; channel; Ca^{2+}-transporting glycoprotein; peptide.

Introduction[2]

Calcium ion controls many enzyme systems (Carafoli and Sottocasa, 1974; Klingenberg, 1970; Carafoli, 1977). Lehninger, Carafoli, and their collaborators revealed the important role of mitochondria in the regulation of Ca^{2+} levels in cytosol. Kinetic and inhibitor analysis suggest the involvement of a

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[2]Abbreviations used: GP, glycoprotein; P, peptide; BLM, bilayer lipid membranes; SDS, sodium dodecylsulfate, PAG, polyacrylamide gel; Rr, ruthenium red; DTT, dithiothreitol; SPAGE, SDS-polyacrylamide gel electrophoresis.

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specific carrier in mitochondrial Ca^{2+} transport (Moore, 1971). Therefore isolation, identification, and reconstruction of ion transport systems are the necessary steps in the investigation of molecular mechanism of Ca^{2+} transport. Attempts to isolate Ca^{2+} carrier have been made in several laboratories (Lehninger, 1971; Gomez-Puyou *et al.*, 1972; Sottocasa *et al.*, 1972; Azarashvili *et al.*, 1978; Jeng *et al.*, 1978; Blondin, 1974).

Work on isolation and identification of Ca^{2+}-transporting substances has been carried out in our laboratory for several years. The component inducing selective Ca^{2+} transport in BLM was isolated from mitochondria and homogenate of different animal and human tissues (Mironova *et al.*, 1973, 1976, 1977). As we have reported recently, Ca^{2+}-transporting properties are due to GP and P present in the preparations (Mironova *et al.*, 1978, 1980). This paper describes the purification procedure of Ca^{2+}-transporting GP and P from heart mitochondria and homogenate as well as their properties and interaction with BLM.

Materials and Methods

Mitochondria were prepared by a conventional procedure. Electrophoresis of proteins in 10% polyacrylamide gel (PAG) was carried out in a Devis system (Davis, 1964), and GP in gel was stained by Schiff reagent (Zacharius *et al.*, 1969) and Coomassie Blue. GP homogeneity was determined using SDS-electrophoresis in 10% PAG. The homogeneity of P was tested using paper chromatography with Filtrak PN-1 in butanol:acetic acid:water mixture (4:1:5) for 24 hr at ambient temperature and stained by ninhydrin. The ion transport activity of isolated substances was investigated using BLM (Mueller *et al.*, 1963). Electrical conductance was measured before and after reconstitution of Ca^{2+} transport in BLM. The experimental cell contained 10 mM Tris-HCl buffer (pH 7.5) with NaCl, KCl (30–100 mM), or $CaCl_2$ (5–10 mM) and aqueous solutions of GP or P. Lipids were isolated from beef brain according to Hara and Radin (1978). Lipid solution (20 mg/ml) in heptane:decane mixture (1:1) was used for BLM formation. Only those samples of lipids which produce BLM with conductance not greater than $1 \cdot 10^{-9}$–$1 \cdot 10^{-8}$ $S \cdot cm^{-2}$ were used throughout the work. Protein was determined according to Lowry *et al.* (1951); thiol groups, by measurement of fluoresceinmercuriacetate fluorescence quenching (Mironov, 1971); sialic acids, according to the Gess procedure (Warren, 1959); and total sugar, with antrone. Proteinase treatment was carried out by the method of Nomoto *et al.* (1960) for 1 hr at 37°C. Inorganic phosphate was measured according to Gerlach and Deuticke (1963). The GP spectrum was registered with a Specord spectrophotometer (GDR). The eluate from Sephadex columns was

analyzed using an LKB Uvicord at 254 nm. The PAG densitogram was taken with a G-II Carl Zeiss Jena microphotometer. The following reagents were used: crystallyzed methylenebisacrylamide (Reanal), Tris (Koch-Light), Coomassie Blue, SDS (Serva), Sephadex G-15, and activated thiol-Sepharose 4B (Pharmacia Fine Chemicals). The ruthenium red (Rr) was a generous gift from Dr. E. Carafoli.

Results

Procedure of Isolation of Ca^{2+}-Transporting Glycoprotein and Peptide

The tissue was extracted with 96° ethanol (pH 7.0)—200 ml of ethanol per 1 g of mitochondrial protein or 5 ml of ethanol per 1 g of beef heart wet tissue. Extraction takes an hour at 4°C under constant stirring. The extract was separated by centrifugation at 5000 × *g* for 15 min at 4°C. The residue was extracted with 50° ethanol in the same proportion as that used in the first step, and the extract was separated similarly. The supernatants were combined and evaporated to dryness under vacuum at 30°C. The resultant dry extract was dissolved in bidistilled water (1 ml per 2 g of wet tissue) and defatted with a chloroform:methanol mixture (2:1) (3 ml of mixture per 1 ml of the extract). The defatted extract was evaporated to dryness at 30°C and dissolved in bidistilled water; then 1.5 ml of solution which corresponded to 100 mg of dry preparation was applied on G-15 Sephadex column (2.8 × 78 cm). The elution was carried out with bidistilled water at the rate of 60 ml/hr. The elution profile of the extract from mitochondria is given in Fig. 1. It consists of seven fractions. All the fractions were evaporated and their ion-transporting properties were tested using BLM. Ca^{2+}-transporting activity was revealed only in fractions 1 and 3. The further purification of fractions

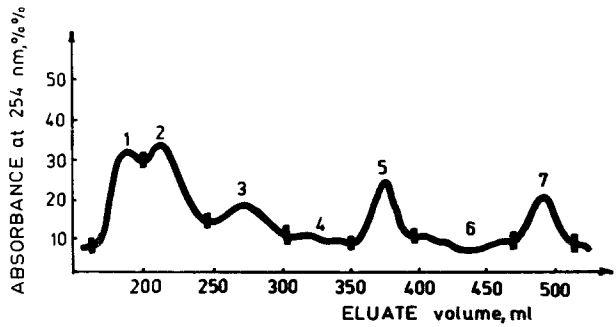

Fig. 1. Typical elution profile of defatted beef heart mitochondria water extract from Sephadex G-15 column (2.8 × 75 cm.). Elution was carried out by distilled water at the rate of 60 ml/hr.

1 and 3 was carried out using paper chromatography. It was shown that the Ca^{2+}-transporting properties of fraction 1 are due to GP and those of fraction 3 are due to P (Mironova *et al.*, 1978, 1980).

In this paper more convenient and appropriate methods of purification of GP and P and their properties are described.

Purification and Physicochemical Properties of Ca^{2+}-Transporting Glycoprotein

The separation of GP from other components of fraction 1 was carried out using gel electrophoresis. Six to seven Coomassie-positive bands of protein nature were revealed in the gel (Fig. 2). Only one of them ($R_f = 0.51 \pm 0.04$) possessed Ca^{2+}-transporting activity. It contains sugar. This band was extracted with bidistilled water and concentrated under vacuum. Finally GP was desalted on the Sephadex G-25 column (1.5 × 43 cm). The GP was running with void volume. Then GP was dialyzed against decationized bidistilled water for 3–5 hr in the cold. The purified GP is homogeneous according to SPAGE (Fig. 3). The molecular weight of GP as determined following Weber and Osborn (1969) is 40,000. GP is water- and 70° ethanol-soluble, stable at ambient temperature for 12–24 hr, and is completely inactivated at 100°C in 3 min. The GP contains 60–70 and 40–30% of

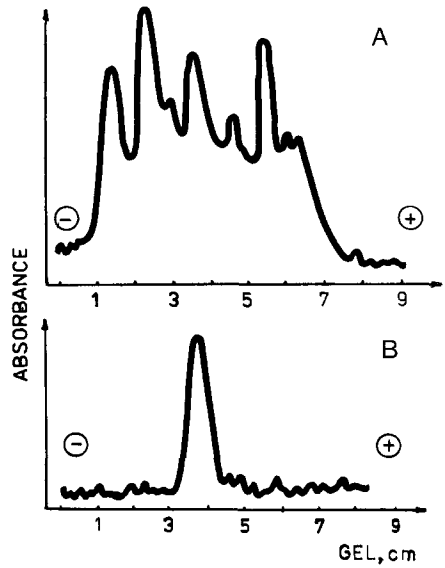

Fig. 2. Densitogram of fraction 1 (A) and of GP (B). 10% PAG, pH 8.3; 0.05 M Tris-glycine buffer, staining by Coomassie blue.

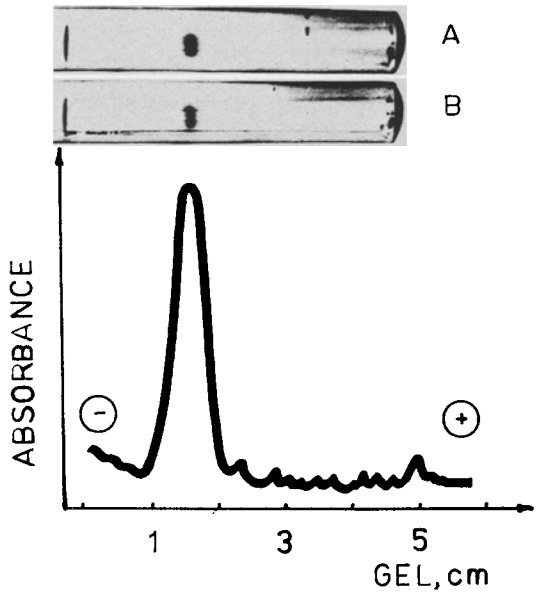

Fig. 3. Densitogram and electrophoregram of GP. 10% PAG, 0.1% SDS, 0.1% 2-mercaptoethanol, pH 7.0, 0.05 M phosphate buffer. (A) Staining by Coomassie blue; (B) staining by Schiff reagent.

protein and carbohydrate, respectively. Sulfur-containing amino acids (1 mole per 1 mole of GP) and sialic acids (2–3 moles per 1 mole of GP) were detected in GP. The amino acid composition of GP is shown in Table I. The yield of GP from 100 g of wet tissue is 0.02–0.1 mg when isolated from mitochondria, and nearly twice as high when isolated from homogenate. The yield depends on the season—the highest amount is obtained in spring, the lowest in autumn. Spring yield may be 6–10 times greater than the autumn yield. Our data on GP and P increase in spring correlate well with spring Ca^{2+} transport acceleration in muscle (Sreter, 1969).

Purification and Physicochemical Properties of Ca^{2+}-Transporting Peptide

Earlier, SH groups of P were revealed (Mironova *et al.*, 1978); therefore, we attempted to use affinity chromatography (Brocklehurst *et al.*, 1973), namely, reversibly binding of thiol-containing P, for the P purification from other components of fraction 3. A 1-g sample of fraction 3 was dissolved in 5 ml of 0.02 M ammonium formate buffer (pH 8.0) and applied on the activated thiol-sepharose 4B column (V = 4 ml) at the rate of 5 ml/hr. The nonbinding components were eluted with 25 ml 0.02 M ammonium formate buffer (pH 8.0) at the rate of 8 ml/hr. The bound P was eluted with 2-mercaptoethanol in

Table I. Amino Acid Composition of Glycoprotein

Amino acids	Number of radicals per molecule initial glycoprotein
1. Asparagine	34.6
2. Threonine	19.8
3. Serine	20.8
4. Glutamine	56.1
5. Proline	14.7
6. Glycine	27.3
7. Alanine	27.6
8. Valine	14.5
9. Methionine	3.2
10. Isoleucine	20.0
11. Leucine	23.2
12. Tyrosine	16.9
13. Phenylalanine	20.6
14. Histidine	8.29
15. Lysine	29.3
16. Arginine	15.0
17. Cysteine	1.12
18. Tryptophan	2.0

the same buffer at the rate of 8 ml/hr for 4 hr. The eluate of P was evaporated to dryness under vacuum at 30°C. The evaporation was repeated three times until complete elimination of mercaptoethanol and buffer. Peptide was dissolved in bidistilled water and applied on the Sephadex G-15 column (2.8 × 45 cm) for separation of 2-thiopyridone.

The rate of elution with water was 60 ml/hr. Peptide was eluted at a volume of 140–200 ml. Pronase treatment of the fraction for 40 min resulted in the loss of Ca^{2+}-transporting activity. Peptide was tested and proved homogeneous using paper chromatography according to the procedure described elsewhere (Mironova *et al.*, 1978). The molecular weight of P is 2000 daltons determined using gel filtration on Sephadex columns. The affinity chromatography step in the purification provides about a 1000-fold rise of P specific activity. Acid hydrolysis revealed 5–6 amino acids, including cysteine and cystine. P is water and ethanol soluble (70°). In water solution the Ca^{2+}-transporting activity of P disappears in 48–72 hr at ambient temperature, and in 3 min at 100°C. The yield of P from 100 g of wet tissue is 0.02–0.05 mg when isolated from mitochondria. The yield depends upon the season in a similar way to GP.

Glycoprotein- and Peptide-Induced Ca^{2+} Permeability of BLM

When GP or P is added to a Ca^{2+}-containing water solution from one or both sides of BLM, the membrane conductance begins to increase rapidly in

1–5 min after the addition of proteins. If the proteins are added to only one of the compartments, the effect develops more slowly and the effective protein concentration is 2–3-fold higher than in the case of two-side addition. The magnitude of conductance change depends upon the GP or P concentration. As can be seen in Fig. 4, the GP concentration vs. BLM conductance curve is sigmoid. The same type of conductance dependence on concentration is observed with P. Both effects are selective with respect to Ca^{2+}. Membrane resistance does not decrease in the presence of K^+ or Na^+ alone, and Mg^{2+} is less effective than Ca^{2+} by an order of magnitude. The GP and P membrane potential at 3-fold Ca^{2+} gradient is equal to 14 ± 0.5 mV, that is, close to the theoretical as calculated for the selective Ca^{2+}-conductance. At threshold concentration of P (1–2 μg/ml) or GP (5–7 μg/ml) in water solution discrete transmembrane current variations are observed. These current fluctuations probably correspond to opening and closing of conductance channels in BLM (Fig. 5). The smallest steps are about 20 pS and some reach 0.5–1.0 nS. The open-state time of channels varies from 1 sec to tens of minutes. The 0.5–1.0-nS channels as well as the 20-pS channels are Ca^{2+} selective. This means that the large channels are aggregates of small Ca^{2+}-selective ones. Rr, the specific Ca^{2+}-transport inhibitor in mitochondria, at 10^{-6}–10^{-5} M induces total closing of BLM channels (Fig. 5). Rr inhibits Ca^{2+}-conductance of BLM in the presence of GP as well at of P (Table II).

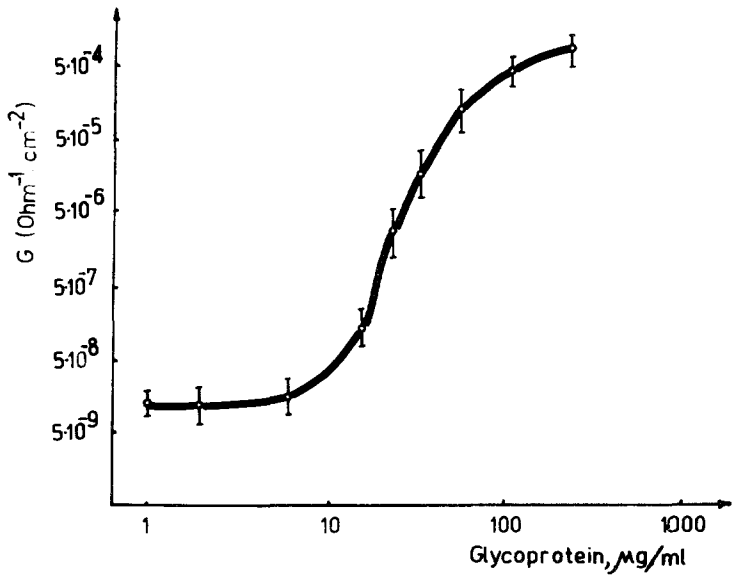

Fig. 4. Dependence of BLM conductance on concentration of GP. The cell contains 10 mM Tris-HCl, pH 7.5, and 5 mM $CaCl_2$.

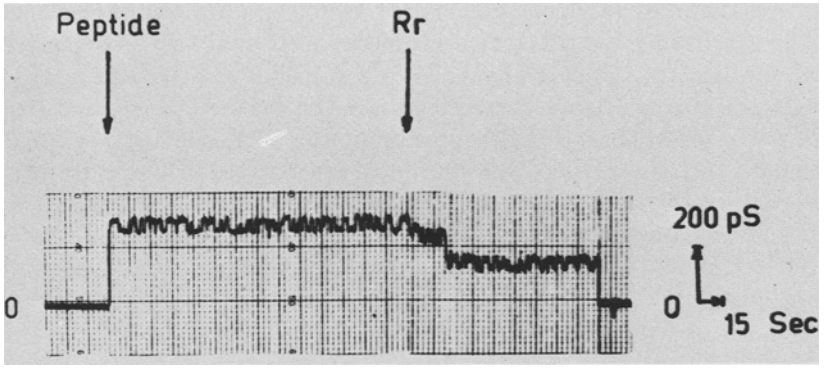

Fig. 5. Influence of Rr on fluctuations of current through the BLM with 3 μg/ml P. 20 mM Tris-HCl, 12 mM $CaCl_2$.

Function of Protein Component in Ca^{2+}-Transporting Glycoprotein

Homogeneous in SDS-PAG, GP contains small amount of lipids. They were detected by the presence of variable amounts of inorganic phosphate (0–13.6 μg/ml protein, from seven measurements) and by staining with 8-anilinonaphthalene-1-sulfonate (Gitler, 1972) and phosphomolybdic acid on silica gel plate (Novitskaya, 1972).

Lipids are considered to provide Ca^{2+} transport through membrane (Evtokienko *et al.*, 1979). To test this possibility, we carried out complete extraction of lipids from glycoprotein and compared the Ca^{2+}-transporting activity of the defatted and initial preparations.

Lipids were removed by a two-step procedure: according to Folch *et al.* (1957) and subsequently according to Blondin (1974). Eluted lipids were analyzed by thin-layer chromatography in silica gel and staining with lipid dyes. Small amounts of neutral lipids (fatty acids, mono-, di-, and triglycerides, cholesterol) and phospholipids [phosphatidylserine (trace) and lysophosphatides] were detected in GP.

The Ca^{2+}-transporting activity of GP was detected after each step of lipid elution (Table III). According to these data the activity of GP did not

Table II. Inhibition of GP-Induced BLM Conductance by Mitochondrial Ca^{2+} Transport Inhibitor[a]

Additions	BLM conductance, $S \cdot cm^2$	Inhibition, %
None	1.2×10^{-9}	—
GP	1.3×10^{-8}	—
GP + 1×10^{-6} M Rr	3.0×10^{-9}	50
GP + 6×10^{-6} M Rr	1.2×10^{-9}	100

[a] Media: 10 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$; 10 μg/ml GP.

Table III. Ca^{2+}-Transporting Activity of GP after Lipid Extraction[a]

Preparation	GP, μg/ml	BLM conductance, $S \cdot cm^{-2}$	Rr inhibition, %
Control (without GP)	—	$(8.2 \pm 1.0) \times 10^{-9}$	—
GP, initial	20	$(7.3 \pm 2.0) \times 10^{-7}$	100
GP after Folch procedure	20	$(6.2 \pm 2.5) \times 10^{-7}$	100
GP after Folch and Blondin procedures	20	$(7.1 \pm 3.1) \times 10^{-7}$	100

[a] Media: 10 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$.

change in the course of lipid extraction, and the inhibitory effect of Rr was not diminished. It may therefore be concluded that lipids are not essential for the Ca^{2+}-transporting activity of GP.

Experiments with proteinase serve as another evidence of the essential role of the protein part of GP for its activity (Table IV). GP activity was completely lost after proteinase treatment, and this was not the result of incubation. Thus the protein is responsible for GP activity, whereas noncovalently bound lipids are obviously not essential for its Ca^{2+}-transporting function.

Essential Role of Thiol Groups for Ca^{2+}-Transporting Activity of the Glycoprotein and Peptide

Thiol groups of GP and P were found to be essential for induction of Ca^{2+} transport through BLM. Thiol-binding reagents—fluoresceinmercuriacetate or mercuriacetate—abolish GP- or P-induced increase of BLM conductance (Table V).

This effect is not due to the hydrophobic influence of the aromatic ring of the dye fluorescein, which is inactive with respect to Ca^{2+}-transport activity.

Dithiothreitol (DTT) restores the activity of P inactivated during storage (Table VI).

As it is shown in Table VI, 1–2 mM of DTT is optimal for P reactivation.

Table IV. Proteinase Treatment Effect on Ca^{2+}-Transporting Activity of GP[a]

Preparation	GP, μg/ml	BLM conductance, $S \cdot cm^{-2}$
Control (without GP)	—	$(6.8 \pm 1.4) \times 10^{-9}$
GP, initial	20	$(5.5 \pm 2.0) \times 10^{-7}$
GP after proteinase treatment	20	$(8.8 \pm 3.2) \times 10^{-9}$
GP after incubation without proteinase	20	$(5.4 \pm 4.2) \times 10^{-7}$
Proteinase after incubation	—	$(6.5 \pm 1.5) \times 10^{-9}$

[a] Media: 10 mM Tris-BCl, pH 7.5; 10 mM $CaCl_2$. Incubation 2 hr at 37°C.

Table V. Influence of Thiol Inhibitors on Reconstituted Ca^{2+} Transport in BLM[a]

Additions	BLM conductance, $S \cdot cm^{-2}$	Inhibition, %
None	1×10^{-9}	—
GP	1.2×10^{-7}	—
GP + 7×10^{-7} M FMA	1.2×10^{-8}	50
GP + 1.4×10^{-6} M FMA	1.0×10^{-9}	100
GP + 8×10^{-7} M HgA	1.1×10^{-8}	50
GP + 2×10^{-6} M HgA	1.2×10^{-9}	100
GP + 2×10^{-6} M (or 2×10^{-5} M F)	1.2×10^{-7}	0

[a]Cell contains 1×10^{-6} M GP; FMA = fluoresceinmercuriacetate; HgA = mercuriacetate; F = fluorescein.

DTT restores the Ca^{2+}-transporting activity of both GP and P, which indicates the essential role of thiol groups for the Ca^{2+}-transporting properties of the substances investigated.

Discussion

GP and P inducing selective Rr-sensitive Ca^{2+} transport through BLM are obtained from beef heart mitochondria using extraction by ethanol. The isolated protein substances differ greatly from other described Ca^{2+} ionophores with respect to their high efficiency as well as their selectivity.

In our opinion the isolation of highly selective and effective ionophores is possible because of the development of new isolation procedures which completely eliminate such reagents as detergents, chaotropic agents, thiol-binding reagents, and proteolytic enzymes. Proteolytic enzyme treatment results in nonstability of protein. Detergents and SH reagents by themselves induce ion transport through BLM (Xengek *et al.*, 1974). As they are usually

Table VI. DTT Restoration of Ca^{2+}-Transporting Activity of GP[a]

Additions	GP, $\mu g/ml$	BLM conductance, $S \cdot cm^{-2}$	Reactivation, %
None	—	8×10^{-10}	—
GP	10	1.5×10^{-8}	100
GP + 2 mM DTT	2	1.3×10^{-8}	500
GP_{in}	10	8×10^{-10}	—
GP_{in} + 0.1 mM DTT	10	2.8×10^{-9}	10
GP_{in} + 1 mM DTT	10	1.1×10^{-8}	47
GP_{in} + 5 mM DTT	10	1×10^{-8}	43
GP_{in} + 10 mM DTT	10	8×10^{-9}	35
GP, freshly prepared	10	2.8×10^{-9}	100

[a]Media: 10 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$. GP_{in} = GP inactivated.

present in the proteins obtained, the measurements of BLM conductivity with detergent-treated preparations should be considered incorrect.

As for the two Ca^{2+}-transporting substances described in our work, we believe P to be an active part of the GP molecule. This conclusion is based on the fact that P is formed from GP. This was observed during prolonged storage of alcohol-defatted extract. Subsequent chromatography showed that a portion of the Ca^{2+}-transporting activity (fraction 1) moved to fraction 3 (Mironova *et al.*, 1978). This means that P can be split from GP during storage and possibly during extraction. Moreover, we isolated P from purified GP (Pronevich *et al.*, 1981). After this separation Ca^{2+}-transporting activity is present in P but not in GP. Thus we are dealing with a glycoprotein–peptide complex where P is the channel-forming part. The nature of the binding of P to GP is being studied at present.

As GP and P are localized in mitochondria and their Ca^{2+}-transporting activity is Rr-sensitive, we suggest that they are the components of the mitochondrial Ca^{2+}-transporting system that provide electrogenic Ca^{2+} transport in the intact mitochondria. Our earlier data showing the presence of glycoprotein in oligomycin-sensitive ATP-ase support this suggestion (Mironova *et al.*, 1976, 1977).

We found that SH groups are essential for the Ca^{2+}-transporting activity of GP and P. This may explain the Ca^{2+}-transport inhibition in mitochondria by SH-group binding reagents. On the other hand, reactivation of the Ca^{2+}-transport activity of GP and P by reducing the previously oxidized SH groups suggests that conversion of SH to S–S may control GP and P activity. This suggestion is in agreement with our earlier observations on changes in sulfur-containing groups in mitochondria during Ca^{2+} transport (Mironov *et al.*, 1971) or ADP phosphorylation (Vinogradov *et al.*, 1966; Mironov, 1971).

The extraction from mitochondria of a GP–P complex inducing selective Ca^{2+} transport in reconstructed systems suggests the following mechanism of Ca^{2+} transport in the intact mitochondria. Ca^{2+} moves through the special channels formed by P, and GP with many Ca^{2+} binding centers creates a high concentration of Ca^{2+} near the channel mouth. The functioning of the channels can be controlled by thio–disulfide transitions of sulfur-containing groups of the glycoprotein–peptide complex.

Acknowledgments

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