ISOLATION OF A SOLUBLE Ca^{2+} BINDING GLYCOPROTEIN FROM OX LIVER MITOCHONDRIA

Gianluigi Sottocasa, Gabriella Sandri, Enrico Panfili, Benedetto de Bernard
Institute of Biological Chemistry, University of Trieste, Italy
and
Paolo Gazzotti, Frank D. Vasington*, and Ernesto Carafoli
Institute of General Pathology, University of Modena, Italy

Received April 14, 1972

SUMMARY: A soluble glycoprotein has been extracted from ox liver mitochondria and purified to a very considerable degree by polyacrylamide gel electrophoresis. Its protein moiety has a molecular weight of about 42,000. It contains about 5 per cent total carbohydrates, including some sialic acid, and up to 30 per cent phospholipids. It binds Ca^{2+} to 2 classes of sites having different affinity. The binding of Ca^{2+} is sensitive to some of the inhibitors of Ca^{2+} binding and transport in mitochondria, among them La^{3+} and ruthenium red.

In the last two years it has become increasingly clear that the energy-linked translocation of Ca^{2+} across the mitochondrial membrane is mediated by a specific carrier or transport system (reviewed in 1). In efforts to extract components of the Ca^{2+} transport system from the membrane, Lehninger (2) has found that osmotic shock releases from rat liver mitochondria a soluble heat-labile factor which is capable of binding Ca^{2+} with high affinity and which is sensitive to La^{3+}.

We have investigated the binding of Ca^{2+} by glycoproteins which we have recently isolated from mitochondria (3,4), prompted by the discovery of Moore that extremely low concentrations of the polysaccharide-stain ruthenium red completely inhibit the energy-linked transport of Ca^{2+} in rat liver mitochondria. On the basis of its Ca^{2+} binding activity we have been able to purify to a very considerable degree a mitochondrial glycoprotein having high affinity for Ca^{2+}. The binding of Ca^{2+} to this glycoprotein is sensitive to inhibitors of respiration-linked Ca^{2+} uptake and high-affinity Ca^{2+} binding, including ruthenium red (5).

The properties of this soluble Ca^{2+} binding glycoprotein are compared with those of an insoluble Ca^{2+} binding factor described in the accompanying paper by Lehninger and his colleagues (6).

* On leave from the Biochemistry and Biophysics Section, Biological Science Group, University of Connecticut, Storrs, Conn., USA.

MATERIALS AND METHODS

Mitochondria were prepared on a large scale from ox liver in 0.25 M sucrose containing 0.05 mM EDTA, which was added because of the extensive use of metal containers. The 10% homogenate, diluted with 1/3 volume of 0.25 M sucrose, was filtered through three layers of nylon net and subjected to differential centrifugation in a "Sharples" centrifuge type 1 A. Microsomal contamination was checked to be less than 7%, and lysosomal contamination was found to be negligible.

To extract the glycolipoprotein the mitochondria were suspended at about 30 mg protein/ml in 10 mM TRIS-phosphate buffer, pH 7.8, and allowed to swell at 0° for 20 min.; they were then diluted with an equal volume of 0.9 M sucrose to induce shrinking. The suspension was then centrifuged at 105,000 x g for 1 hr, and the resulting supernatant fraction subjected to free-phase electrophoresis on which the glycoproteins set free in solution by swelling migrate as the fastest-moving component in agreement with previous data (7,8). This process resulted in a selective movement of this component toward the anode. The electrophoretic system consisted of a U-shaped glass tube. One branch was equipped with a cooling jacket. The free branch and the loop were filled with 1.12 M sucrose and 0.035 M TRIS chloride pH 6.7. In the cooled portion of the tube 200 ml of the supernatant fraction was layered; the rest of the tube was filled with 0.01 M TRIS phosphate, pH 7.8.

The anode was immersed into the sucrose-containing branch and the catode into the other. After 2 hrs at 1000 volts and 4 - 5 mA a clear greenish band became visible at the interface between the sucrose and the sample solution. This band, collected and adjusted to pH 5.5 with 1/10 volume of 0.355 M TRIS phosphate was subjected to preparative polyacrylamide gel electrophoresis on a simple apparatus based on the principle described by Mann and Huang (9), using two layers of polyacrylamide gel of 10 ml each, at 3 and 10% respectively. The buffer in the running gel was at pH 7.5 according to Williams and Reisfeld (10); no spacer gel was used. Bands were displaced from the column by a stream of TRIS-veronal, pH 7.0 and monitored for protein by continuous recording of absorbance at 280 nm (UV-cord LKB, Sweden). The fraction containing the first peak that appeared was collected, lyophilized, and stored at -20°.

Neutral sugars were determined according to Scott and Melvin (11), hexosamines after 6 hrs hydrolysis in 6N HCl at 105° under nitrogen by the Elson-Morgan reaction (12), and sialic acid after 2 hrs hydrolysis in 0.1 N H_2SO_4 at 80°, according to Warren (13). Ca^{2+} binding ability of the glycoprotein was determined by the Chelex method (14) which was convenient because of its rapidity, but gave somewhat variable results. The final experiments

were therefore always carried out by using micro-equilibrium dialysis (15) or the flow-dialysis method of Colowick and Womack (16).

RESULTS

In preliminary runs on polyacrylamide gel, a major glycoprotein band was identified by its metachromatic reaction with toluidine blue and alcian blue, or by reaction with ruthenium red. This band could also be stained in the gels with murexide, indicating the presence of Ca^{2+}. Under the conditions of preparative electrophoresis described above, this glycoprotein traveled 12 cm in about 5 hrs, and was recovered as a very sharp symmetrical peak. M.W. estimates (gel electrophoresis in the presence of SDS according to Weber and Osborne (17)) yielded a figure of about 42,000. The carbohydrates present in the molecule were found to be sialic acid, hexosamines (mainly galactosamine and glucosamine) and neutral sugars (mainly glucose and xylose) (5.7, 18 and 33 μgs/mg protein, respectively, when measured as described in the Methods. GLC measurements, however, seem to yield high levels. The matter is currently under study.). Variable amounts of phospholipids were also present (up to 300 μgs/mg protein) and could be removed with chloroform-methanol. As already indicated by the positive reaction with murexide, the protein contained a variable amount of Ca^{2+} and, in addition, Mg^{2+}, both up to

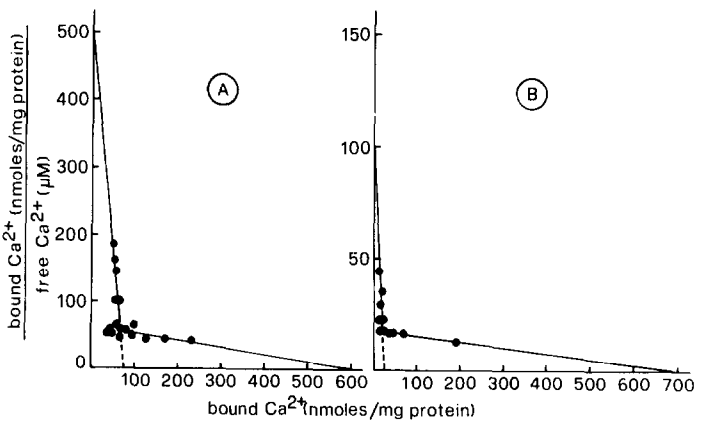

Figure 1A. Scatchard plot of calcium binding to the purified glycoprotein. Experimental conditions: Ca^{2+} binding ability has been determined by flow dialysis according to Colowick and Womack (18). The upper part of the cell contained in 2 ml 0.230 mg purified glycoprotein, 5 mM Tris-glycine buffer (pH 8.3). Approximately 3 mM veronal Tris-buffer was also added with the protein and the final pH was corrected to 8.3 by addition of Tris base. The flow rate in the lower part of the chamber (volume 0.1 ml), was 0.5 ml/minute.

Figure 1B. Scatchard plot of Ca^{2+} binding to the purified glycoprotein prepared in the absence of EDTA. Experimental conditions as in Fig. 1A. Protein content of the upper part of the cell was 0.280 mg in 2 ml.

400 nmoles per mg. When tested for Ca^{2+} binding ability, the glycoprotein was found to be very active. As indicated by Figure 1A, a biphasic Scatchard plot is obtained. The high-affinity leg of the curve may be extrapolated in the vicinity of 72 nmoles of Ca^{2+} bound per mg protein with a dissociation constant of the order of 10^{-7} M. The low affinity leg, on the other hand, intercepts the ascissa at about 600 nmoles of Ca^{2+} per mg/protein, with a dissociation constant of the order of 10^{-5} M. Since the molecular weight of the glycoprotein has been determined to be about 42,000 it follows that 1 mole binds approximately 3 moles of Ca^{2+} with high affinity.

The isolated glycoprotein, which corresponds to 0.5 - 1% of the total mitochondrial protein, may account for all the high-affinity sites of intact mitochondria. As shown by Figure 1B, omission of EDTA during the preparation results in a decrease of the total number of high affinity sites, but no change in affinity suggesting that EDTA protects Ca^{2+} binding activity. As indicated in Figure 1, EDTA is not required for the binding of Ca^{2+}. However, minute amounts of EDTA from the isolation medium were found to remain bound to the glycoprotein throughout the purification steps.

Table 1 shows the effect of some inhibitors on the binding of Ca^{2+} by the isolated glycoprotein. La^{3+} inhibited both the high- and low-affinity

TABLE 1

Effect of various inhibitors on high- and low-affinity binding of Ca^{2+} to the soluble glycoprotein. In these 2 experiments the binding has been determined with the Chelex method. Concentrations: glycoprotein, 21 μgs in expt. 1, 23 μgs in expt. 2. $LaCl_3$, 10 μM. Ruthenium red, 25 μM. Butacaine, 100 μM. The heat lability of the protein was tested after incubation for 60 minutes at 100° C (expt. 1) or for 120 minutes at 100° C (expt. 2).

EXP.	INHIBITOR	BOUND CA^{2+} (nMOLES/MG PROTEIN)*	
		HIGH-AFFINITY BINDING	LOW-AFFINITY BINDING
1	None	18	106
	La^{3+}	0	0
	Ruthenium red	11.8	89
	Butacaine	17.7	116
	Boiling	-	49.5
2	None	46	85
	La^{3+}	0	0
	Ruthenium red	12	23.5
	Butacaine	15	42
	Boiling	-	12.5

* The high-affinity binding was tested in the presence of 0.5 nmoles (expt. 1) and 1.5 nmoles (expt. 2) of Ca^{2+}, the low-affinity binding in the presence of 5 nmoles.

sites 100%; ruthenium red also inhibited both sites, but only about 50%. An inhibitor of mitochondrial low-affinity binding, butacaine (18), had only a slight inhibitory effect, a somewhat surprising finding in view of the large amount of phospholipids present in the preparation. It must be pointed out, however, that in some preparations the inhibition by butacaine was more pronounced and was particularly evident against the low affinity sites. As indicated in Table I, the binding to both classes of sites was moderately heat-labile; heating of the preparation at 100° C for 1 hour induced an inhibition of about 50%, and only after very prolonged incubations at 100° C could higher levels of inhibition be attained. Other mitochondrial inhibitors (dinitrophenol, oligomycin, valinomycin, antimycin A) had no effect on the binding of Ca^{2+}.

DISCUSSION

The soluble glycoprotein described in the present paper has a very high affinity for Ca^{2+}, comparable to that of the high-affinity binding sites in intact mitochondria (19). It is also sensitive to some of the agents which inhibit the transport and the binding of Ca^{2+} in intact mitochondria. Of special interest is the inhibitory effect of ruthenium red, which we have found recently to abolish high-affinity binding in mitochondria (20). The protein contains also a large number of low-affinity Ca^{2+} binding sites; although it would seem logical to relate them to the low-affinity sites in intact mitochondria, the modest and variable effect of butacaine does not permit their identification with the phospholipids of the glycoprotein molecule, whereas the complete inhibition by La^{3+} makes them different from the low-affinity sites of intact mitochondria. More detailed investigations on this point are presently under way.

The results presented indicate that the glycoprotein fraction may contain the high-affinity Ca^{2+} binding sites of the mitochondrial membranes. Although it is tempting to suggest that it represents a specific Ca^{2+}-carrier involved in the translocation of Ca^{2+} across the mitochondrial membrane, at the present stage we intend to be very cautious on this point. The soluble glycoprotein described in this study has some similarities and also some differences with respect to the insoluble Ca^{2+} binding factor of Gomez-Puyou et al. (6); these are summarized in the latter paper. It is also very different in properties from the Ca^{2+} binding (Calsequestrin) recently isolated by McLennan and Wong from sarcoplasmic reticulum (21). Similarly to calsequestrin and to other Ca^{2+}-binding proteins, preliminary amino acid analyses have however shown the presence of a very high number of aspartic and glutamic acid residues. The glycoprotein on the other hand resembles the estrogen-induced, high molecular weight, Ca^{2+}-binding glycolipophosphoprotein recently isolated

from toad serum by Ansari et al. (22).

Studies of the possible Ca^{2+}-induced conformational changes of the glycoprotein and the preparation of a specific antiserum, are now in progress.

ACKNOWLEDGMENTS

The research has been supported by the Italian National Research Council (Contract No. 6902109-115.3453.0), by the University of Connecticut Research Foundation, and by the NATO Research Program (Grant No. 522). The expert technical assistance of Mrs. Daniela Ceccarelli-Stanzani and Mr. B. Gazzin is gratefully acknowledged.

REFERENCES

1. Lehninger, A. L., Biochem. J. 119, 129 (1970).
2. Lehninger, A. L., Biochem. Biophys. Res. Commun. 42, 312 (1971).
3. de Bernard, B., Pugliarello, M. C., Sandri, G., Sottocasa, G. L., and Vittur, F., FEBS Letters 12, 125 (1971).
4. Sottocasa, G. L., Sandri, G., Panfili, E., and de Bernard, B., FEBS Letters 17, 100 (1971).
5. Moore, C. L., Biochem. Biophys. Res. Commun. 42, 298 (1971).
6. **Gomez-Puyou, A., Tuena de Gomez-Puyou, M., Becker, G., and Lehninger, A. L., Biochem. Biophys. Res. Commun., 47, 814 (1972).**
7. Sottocasa, G. L., Sandri, G., Panfili, E., and de Bernard, B., in Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E., and Siliprandi, N., Eds., Biochemistry and Biophysics of the Mitochondrial Membrane, Academic Press, New York (1972), in press.
8. Carafoli, E., Gazzotti, P., Vasington, F. D., Sottocasa, G. L., Sandri, G., Panfili, E., and de Bernard, B., in Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E., and Siliprandi, N., Eds., Biochemistry and Biophysics of the Mitochondrial Membrane, Academic Press, New York (1972), in press.
9. Mann, M. B. and Huang, P. C., Anal. Biochem. 32, 138 (1969).
10. Williams, D. E. and Reisfeld, R. A., Ann. N. Y. Acad. Sci. 121, 373 (1964).
11. Scott, T. A. and Melvin, E. H., Anal. Biochem. 25, 1656 (1953).
12. Elson, L. A. and Morgan, W. T. J., Biochem. J. 27, 1882 (1933).
13. Warren, L., J. Biol. Chem. 234, 1971 (1959).
14. Wasserman, R. H. and Taylor, A. N., Science 152, 791 (1966).
15. Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A., J. Biol. Chem. 244, 3038 (1969).
16. Colowick, S. P. and Womack, F. C., J. Biol. Chem. 244, 774 (1969).
17. Weber, K. and Osborne, M., J. Biol. Chem. 244, 4406 (1969).
18. Rossi, C., Azzi, A. and Azzone, G. F., J. Biol. Chem. 242, 951 (1967).
19. Reynafarje, B. and Lehninger, A. L., J. Biol. Chem. 244, 584 (1969).
20. Vasington, F. D., Gazzotti, P., Tiozzo, R., and Carafoli, E., Biochim. Biophys. Acta 256, 43 (1972).
21. McLennan, D. H. and Wong, P. T. S., Proc. Nat. Acad. Sci. 68, 1231 (1971).
22. Ansari, A. Q., Dolphin, P. J., Lazier, C. B., Munday, K. A., Biochem. J. 122, 107 (1971).