A SOLUBLE, HEAT-LABILE, HIGH-AFFINITY CA^{2+}-BINDING FACTOR EXTRACTED FROM RAT LIVER MITOCHONDRIA

Albert L. Lehninger

Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, Maryland 21205

Received December 15, 1970

Summary. Rat liver mitochondria exposed to osmotic shock (distilled water) lose the capacity for high-affinity binding of Ca^{2+}. The Ca^{2+} binding activity can be recovered in soluble form in the extract. It is heat-labile, not affected by uncouplers or respiratory inhibitors, and is inhibited by Sr^{2+}, Mn^{2+}, La^{3+}, but not by Mg^{2+}. The particle weight of the soluble factor exceeds 150,000.

Rat liver mitochondria contain both high-affinity and low-affinity binding sites for Ca^{2+} (1). The high-affinity sites, which are small in number, have been postulated to be the ligand binding sites of a specific Ca^{2+} transport system in the membrane (1,2). Such high-affinity Ca^{2+} binding sites occur in all vertebrate mitochondria tested (2,3), in parallel to their capacity for energy-linked accumulation of Ca^{2+} from very low concentrations (2,3). However, they are absent in mitochondria from yeast (4) and blowfly muscle (5), which are unable to accumulate Ca^{2+} from low external concentrations. The high-affinity Ca^{2+} binding sites are localized in the inner mitochondrial membrane (6).

This paper describes the extraction and some properties of a soluble, heat-labile fraction from rat liver mitochondria which appears to contain the high-affinity Ca^{2+}-binding sites.

Extraction of soluble high-affinity Ca^{2+}-binding activity. Earlier work showed that exposure of rat liver mitochondria to distilled water caused loss of high-affinity Ca^{2+}-binding activity (1), as well as loss of the capacity to accumulate Ca^{2+} in the absence of ADP and Mg^{2+} (7). Since similar hypotonic treatment has been found to release certain binding and/or transport proteins from bacteria (8), such as those for amino acids and sulfate (reviewed by Kaback (9)), a search was made for high-affinity Ca^{2+}

binding activity in the water-soluble fraction resulting when rat liver mitochondria were exposed to distilled water at 0° and then removed by centrifugation (7). Assay of Ca^{2+} binding activity by the mitochondrial extract was carried out by equilibrium dialysis in cellophane bags against various initial concentrations of $^{45}Ca^{2+}$, followed by measurement of the radioactivity in the inside and outside compartments. The results of a typical experiment are given in the form of a Scatchard plot in Figure 1. The mitochondrial extract yielded a biphasic Scatchard plot closely resembling the binding curve given by intact rat liver mitochondria (1). The number and the affinity of the high-affinity Ca^{2+}-binding sites of the mitochondrial extract are not greatly different from those of intact rat liver mitochondria (Table 1). The "ghost" or water-shocked mitochondria remaining after water extraction retained no high-affinity Ca^{2+} binding activity, as was shown earlier (1).

Since atomic absorption spectrometry showed that the mitochondrial extracts contain about 2 nmoles endogenous Ca^{2+} per mg protein, which is nearly sufficient to saturate the high-affinity sites, the specific radioactivity of the added Ca^{2+} was corrected for the endogenous unlabeled Ca^{2+}. Direct measurements indicated that the added isotopic Ca^{2+} mixed completely with the endogenous unlabeled Ca^{2+}. Subsequent assays of high-affinity Ca^{2+} binding by mitochondrial extracts were carried out with the micro-equilibrium

Table 1. Comparison of Ca^{2+} binding sites in intact mitochondria and in extracts. The dissociation constants (K) are given in μmoles per liter and the number of sites (n) as nmoles per mg protein.

	High-affinity sites		Low-affinity sites	
	K	n	K	n
Intact RLM	1.2 ± 0.4	3.0	52	30
Extracts of RLM	1.6 ± 0.5	1.47	690	133

dialysis cells and apparatus described by Englund et al. (10), which permitted measurements on 20 μl volumes of extract containing 10-20 μg of protein.

Table 1 compares the dissociation constants and the number of both the high- and low-affinity Ca^{2+} binding sites of a series of 19 extracts of rat liver mitochondria with the corresponding values obtained on intact rat liver mitochondria (1). K_{diss} for the high-affinity sites of the extracts remained nearly constant when the protein concentration was varied over a nine-fold range, indicating that the equilibrium of Ca^{2+} binding is given by the general equation

$$Ca^{2+} + A^{2-} \rightleftharpoons CaA$$

and that the binding sites are non-cooperative.

Other extraction procedures. Extraction of freshly isolated intact rat liver mitochondria with cold 0.3 M sucrose yielded no soluble high-affinity Ca^{2+} binding activity (Figure 1), indicating that hypotonic conditions are necessary for extraction of the Ca^{2+} binding activity. Sonication of rat liver mitochondria released some soluble Ca^{2+} binding activity, but this had a very low specific activity. Of all procedures examined, water extraction proved to give the largest yield and highest specific activity of high-affinity Ca^{2+} binding activity, exceeding 60 per cent of that present in intact mitochondria.

General properties of the soluble Ca^{2+} binding factor. The soluble Ca^{2+}-binding activity of mitochondrial extracts prepared in the manner described above did not pass through cellophane membranes. No activity was lost on dialyzing as long as 18 hours against Tris buffer at pH 7.4; normally, the equilibrium dialysis measurements required only about 4 hours. The high-affinity Ca^{2+} binding activity of undialyzed extracts survived freezing and thawing with no loss in activity; samples of the water extract of rat liver mitochondria appeared to retain full activity after several weeks in the frozen state. Heating of the mitochondrial extract for 10 min in a boiling

water bath caused complete loss of soluble Ca^{2+}-binding activity.

Effect of Sr^{2+}, Mn^{2+}, Mg^{2+} and La^{3+}. Intact rat liver mitochondria accumulate Ca^{2+}, Sr^{2+}, and Mn^{2+} during coupled respiration, but do not accumulate Mg^{2+} (11); correspondingly, they show high-affinity binding of Ca^{2+}, Sr^{2+}, and Mn^{2+}, but not of Mg^{2+} (1). Data in Figure 2 show that both Sr^{2+} and Mn^{2+} at low concentrations (10 μM) substantially inhibited high-affinity binding of Ca^{2+} when the latter was present at 10 μM, presumably by competing with Ca^{2+}. However, Mg^{2+} gave no inhibition of high-affinity Ca^{2+} binding at 10 μM and inhibited only slightly at 2 mM. On the other hand, Mg^{2+} at high concentrations competes with Ca^{2+} for binding to the low-affinity sites, as is also true in intact mitochondria (1). Therefore the addition of a high

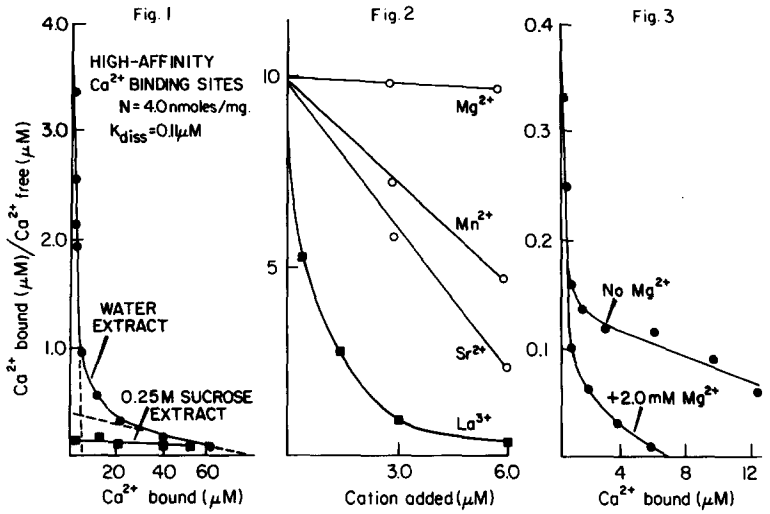

Figure 1. Scatchard plots of equilibrium dialysis data on Ca^{2+} binding by extracts of rat liver mitochondria. The dialysis medium contained 0.25 M sucrose and 2.0 mM Tris Cl pH 7.4; the extracts were added at 1.0 mg protein per ml. The water extract was prepared by suspending freshly isolated rat liver mitochondria in cold distilled H_2O at 20 mg mitochondrial protein per ml for 15 min at 0° followed by centrifugation at 40,000 for 30 min.

Figure 2. Effect of Mg^{2+} on Ca^{2+} binding by water extract. Details as in Figure 1.

Figure 3. Effect of divalent cations and La^{3+} on high-affinity Ca^{2+} binding. Equilibrium dialysis was carried out in medium of 0.25 M sucrose + 2.0 mM Tris Cl, using 2.0 μM $^{45}Ca^{2+}$ and 1.0 mg protein per ml. The pH was 7.4.

concentration of $MgCl_2$ (2 mM) to the mitochondrial extracts has the affect of greatly decreasing low-affinity Ca^{2+} binding while leaving high-affinity Ca^{2+} binding activity intact, a property which simplifies assay of high-affinity Ca^{2+} binding by allowing the "swamping" of the low-affinity Ca^{2+}-binding sites with Mg^{2+} (Figure 3).

La^{3+} and other rare earth cations inhibit the high-affinity binding of Ca^{2+} to intact mitochondria (12) and interfere with the transport of Ca^{2+} into mitochondria (12-15). La^{3+} also inhibits high-affinity binding of Ca^{2+} by the soluble mitochondrial extracts (Figure 2).

Effect of uncoupling agents and other inhibitors. High-affinity Ca^{2+} binding by intact mitochondria is inhibited by 2,4-dinitrophenol and other uncoupling agents of oxidative phosphorylation, but not by respiratory inhibitors, by oligomycin, or by valinomycin (1). None of these agents inhibit Ca^{2+} binding at the high-affinity sites in the soluble mitochondrial extracts. The failure of 2,4-dinitrophenol to inhibit Ca^{2+} binding in the extracts is not unexpected, since inhibition of Ca^{2+} binding by uncoupling agents in intact mitochondria has been attributed to the capacity of these agents to allow H^+ to pass through the mitochondrial membrane in exchange for Ca^{2+} (1).

High-affinity Ca^{2+} binding in the extracts was not inhibited by p-chloromercuribenzoate, iodoacetamide, or by mersalyl.

Appropriate molecular weight. In order to ascertain the approximate size of the Ca^{2+} binding factor in the mitochondrial extracts they were fractionated on Sephadex gel columns (experiments carried out by Dr. Morley Hollenberg). The Ca^{2+} binding activity was not retarded on passage of the extracts through G-25, G-75, or G-100 Sephadex columns. Retardation did however occur on a Sephadex G-200 column; a nearly symmetrical elution peak was observed. The elution volume suggested a particle weight of at least 150,000 daltons, greatly exceeding the molecular weights of the sulfate-binding protein of S. typhimurium, about 32,000 (16), the various amino acid binding proteins of E. coli, about 36,000 (reviewed by Kaback (9)), and the

Vitamin D-stimulated Ca^{2+} binding protein of chicken small intestine, which has a molecular weight of about 28,000 (17).

Discussion. The properties of the soluble high-affinity Ca^{2+} binding factor in the mitochondrial extracts are comparable in all respects examined to those of the high-affinity Ca^{2+} binding sites of intact rat liver mitochondria. Its Ca^{2+}-binding capacity has essentially the same ligand affinity, specificity, and response to inhibitors, including La^{3+}, which has been suggested to be a specific inhibitor of mitochondrial Ca^{2+} transport (14,15). The observations reported here, taken together with earlier data, strongly suggest that the soluble Ca^{2+} binding factor described here represents the ligand-binding portion of a specific Ca^{2+} carrier or transport system of the inner mitochondrial membrane. Whether one molecule of the soluble factor binds several or many Ca^{2+} ions and whether the Ca^{2+} binding factor is a relatively small molecule which is attached to a high-molecular weight membrane protein remain to be established.

Further work on the purification, molecular weight, and chemical nature of the Ca^{2+} binding factor is under way, as are efforts to reconstitute Ca^{2+} transport in transport-negative mitochondrial preparations.

Acknowledgements. This study was supported by grants from the National Institutes of Health (GM 05919) and the National Science Foundation (GB 7032). The technical assistance of Paulette Riley and the advice and help of Drs. Ernesto Carafoli, Morley Hollenberg, and Chung-ho Chen are gratefully acknowledged.

References

1. Reynafarje, B. and Lehninger, A. L., J. Biol. Chem., 244, 584 (1969).
2. Lehninger, A. L., Biochem. J., 119, 129 (1970).
3. Carafoli, E. and Lehninger, A. L., Biochem. J., In press.
4. Carafoli, E., Balcavage, W. X., Lehninger, A. L., and Mattoon, J. R., Biochim. Biophys. Acta, 205, 18 (1970).
5. Carafoli, E., Hansford, R., Sacktor, B., and Lehninger, A. L., J. Biol. Chem., In press.
6. Carafoli, E. and Gazzotti, P., FEBS Letters, In press.
7. Caplan, A. I. and Greenawalt, J. W., J. Cell Biol., 244, 584 (1968).
8. Heppel, L., Science, 156, 1451 (1967).
9. Kaback, H. R., Ann. Rev. Biochem., 39, 561 (1970).

10. Englund, P., Huberman, J. A., Jovin, T. M., and Kornberg, A., J. Biol. Chem., 244, 3038 (1969).
11. Lehninger, A. L., Carafoli, E., and Rossi, C. S., Adv. in Enzymol., 29, 259 (1967).
12. Lehninger, A. L. and Carafoli, E., Arch. Biochem. Biophys., Submitted.
13. Lehninger, A. L., Ann. N.Y. Acad. Sci., 147, 816 (1969).
14. Mela, L., Arch. Biochem. Biophys., 123, 286 (1968).
15. Chance, B., Azzi, A., and Mela, L., in Symposium on the Molecular Basis of Membrane Function, Ed. D. C. Tosteson, Prentice-Hall, Englewood Cliffs, 1969, p. 561.
16. Pardee, A. B., Science, 156, 1627 (1967).
17. Wasserman, R. H., Corradino, R. A., and Taylor, A. N., J. Biol. Chem., 243, 3978 (1968).