Biochimica et Biophysica Acta, 347 (1974) 160–167

BBA 46741

ENERGY-DEPENDENT ACCUMULATION OF IRON BY ISOLATED RAT LIVER MITOCHONDRIA

III. SUBMITOCHONDRIAL LOCALIZATION OF THE IRON ACCUMULATED

INGE ROMSLO and TORGEIR FLATMARK

Department of Biochemistry, University of Bergen, Årstadvollen, Bergen (Norway)

(Received November 6th, 1973)

SUMMARY

Isolated rat liver mitochondria accumulate iron partly by an energy-dependent and partly by an energy-independent mechanism (Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 305, 29–40). When the iron-loaded mitochondria were disrupted mechanically and the mitochondrial subfractions isolated by density gradient centrifugation, the iron accumulated by the energy-dependent mechanism was recovered mainly in the soluble matrix and intermembrane space (approx. 50% of the total activity) and the inner membrane (approx. 30%). A negligible contribution to the total iron content of the soluble fraction by intermembrane space was revealed by the preparation of 'mitoplasts'. On the other hand, most of the energy-independent iron accumulation was confined to the outer and inner membranes (approx. 35% of the total activity in each).

INTRODUCTION

In previous studies from this laboratory, an energy-dependent accumulation of iron by isolated rat liver mitochondria has been described [1, 2]. Our studies so far point to the presence of a carrier-mediated transport of iron across the inner membrane similar to that of Ca^{2+} [3–5]. Thus, the energy-dependent accumulation of iron was inhibited by uncouplers, La^{3+}, ruthenium red and Ca^{2+} [6]. Furthermore, iron had a marked influence on the oxidative metabolism of the mitochondria, inducing a transient stimulation of State 4 as well as State 3 respiration which was proportional to the amount of iron added [7, 8]. Concomitant to the stimulation of respiration, an increase in the rate of oxidative phosphorylation was observed, although the P/O ratio was slightly reduced [7, 8]. Furthermore, iron induced a transition of the redox state of the cytochrome *b* complex towards oxidation, a lowering of the respiratory control ratio with ADP and an increase in the Mg^{2+}-stimulated ATPase [8].

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

The present paper deals with the submitochondrial localization of the iron accumulated by the two processes. Data are given which indicate that the iron accumulated by the energy-dependent mechanism is recovered in the matrix and inner membrane fractions, whereas the iron accumulated by the energy-independent mechanism is confined to the outer and inner membranes.

A preliminary account of this work has already appeared [8].

MATERIALS AND METHODS

Preparation of mitochondria and iron accumulation experiments were performed as previously described [6].

Isolation of the mitochondrial subfractions

To study the submitochondrial localization of the iron accumulated, the iron-loaded mitochondria were subjected to density gradient centrifugation essentially as described [2]. The mitochondrial band thus obtained was sucked off, resuspended in the incubation medium and re-centrifuged at a time integral of $(\text{rev./min})^2$ equal to $270 \cdot 10^7 \cdot \text{min}^{-1}$ in the swinging-bucket HB-4 rotor of Sorvall RC 2-B refrigerated centrifuge (R_{max} and R_{min} were 13.9 and 6.1 cm, respectively). The final pellet was fractionated by two different procedures.

Procedure A. The mitochondria were fractionated essentially as described by Wojtczak and Sottocasa [9]. Following the osmotic shock, the mitochondrial suspension (20–30 mg of protein per ml) was subjected to ultrasonic vibration. The sonifier (Branson Sonifier, Model S-75) was operated at a current output of 4 A using a rosett cooling cell (vol. 6 ml) cooled in ice water, thus keeping the temperature in the cell always below 11 °C (on an average below 6 °C). At these conditions a sonication time of 90 s (three times for 30 s) was required to ensure optimal disintegration and purity of the mitochondrial subfractions. The sonicated mitochondria were diluted with an equal volume 0.15 M KCl, and 15 ml of the suspension thus obtained were layered on a discontinuous density gradient of sucrose (from bottom to top: 5 ml 1.6 M sucrose with 5% (w/v) Ficoll, 7 ml of 1.17 M sucrose and 5 ml of 0.75 M sucrose) and centrifuged at a time integral of $(\text{rev./min})^2$ equal to $104 \cdot 10^9 \cdot \text{min}^{-1}$ in a swinging bucket rotor (Spinco SW 25). The soluble intermembrane space and matrix constituents were found in the upper suspension medium. Outer membranes, in part contaminated by soluble intermembrane space and matrix constituents, were recovered as two separate bands, i.e. one faint band at the interphase suspension medium–0.75 M sucrose and the main outer membrane fraction at the interphase 0.75–1.17 M sucrose. The inner membranes were recovered at the interphase 1.17 M –1.60 M sucrose. Insoluble iron polymers were recovered at the bottom of the tube ($< 2\%$ of the total amount of iron applied on the gradient). The fractions were sucked off [2] and diluted with appropriate volumes of the incubation medium for counting of radioactivity [2], enzymic assays and protein determination (see below).

Procedure B. The iron-loaded mitochondria were fractionated following an incubation with digitonin essentially as described by Chan et al. [10], except that the concentration of digitonin in most experiments was higher, i.e. 0.2 ± 0.05 mg per mg of mitochondrial protein. After removal of the outer membrane and the intermembrane

space constituents with digitonin (fraction L_d), the 'mitoplasts' (inner membranes plus matrix) were further fractionated with Lubrol WX (0.16 mg detergent per mg of protein) in inner membrane fraction (H_d) and matrix fraction (S_d) [10].

Enzymic assays

Amine oxidase (EC 1.4.3.4) was assayed as described by Aas [11], cytochrome *c* oxidase (EC 1.9.3.1) as described by Yonetani and Ray [12], malate dehydrogenase (EC 1.1.1.37) as described by Bergmeyer [13] and adenylate kinase (EC 2.7.4.3) as described by Schnaitman and Greenawalt [14]. When fractionated according to Chan et al. [10], all enzymic assays were performed in the presence of Lubrol WX [14].

Protein was determined by the Folin-Ciocalteau reagent [15] after appropriate dilution of the fractions to avoid interference by sucrose in the assay [16].

Chemicals

ADP, digitonin, Lubrol WX, NADP (sodium salt), NADH (disodium salt, grade III), hexokinase (Type III from yeast) and glucose-6-phosphate dehydrogenase (Type XV from baker's yeast) were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (A grade) was purchased from Calbiochem (Luzern, Switzerland), $^{59}FeCl_3$ was obtained from Institutt for Atomenergi, Kjeller, Norway, and [1-^{14}C]tyramine hydrochloride from the Radiochemical Centre, Amersham, England.

Other chemicals were of the highest purity commercially available. Double quartz distilled and deionized water was used throughout.

RESULTS

Two methods have been employed to isolate subfractions of iron-loaded mitochondria. Both methods, i.e. that based on chemical disruption with digitonin and that based on mechanical disruption had to be slightly modified from the original procedures [9, 10]. Thus, the digitonin concentration required for the removal of the outer membrane was approx. two times higher than that required for native mitochondria (0.10–0.12 mg digitonin per mg of protein). This increased resistance towards digitonin is probably due to changes imposed on the outer membrane by the accumulation of iron (as iron–sucrose complex(es) [6]). Unfortunately, this rather high digitonin concentration resulted in slight damage to the inner membrane as revealed by a leakage of matrix enzymes e.g. malate dehydrogenase (Fig. 2). Furthermore, it should be noted that the iron-loading slightly changes the sedimentation profile of the membrane fractions on density gradient centrifugation as compared to those of native mitochondria (Slinde, E., unpublished).

Enzyme characterization of mitochondrial subfractions

The total and specific activities of the chosen marker enzymes in the various mitochondrial subfractions, obtained by mechanical disruption, are shown in Table I and Fig. 1. As expected, the adenylate kinase and malate dehydrogenase activities were recovered mainly in the soluble fraction (S_m), whereas the cytochrome *c* oxidase activity was confined to the inner membrane fraction (H_m). The amine oxidase activity was recovered in two fractions (L_mI and L_mII). The minor fraction (L_mI),

TABLE I

PROTEIN CONTENT, TOTAL ACTIVITIES AND SPECIFIC ACTIVITIES OF MARKER ENZYMES IN MITOCHONDRIAL SUBFRACTIONS

Rat liver mitochondria were disrupted mechanically (swelling, shrinking and sonication) and the subfractions were isolated by discontinuous density gradient centrifugation (for details, see Materials and Methods). S_m, soluble fraction (matrix+intermembrane space) recovered in the upper part of the density gradient; L_mI, light membrane fraction (outer membrane) recovered at the interphase incubation medium–0.75 M sucrose; L_mII, light membrane fraction (outer membrane) recovered at the interphase 0.75 M–1.17 M sucrose; H_m, heavy membrane fraction (inner membrane) recovered at the interphase 1.17 M–1.60 M sucrose. The result represent the mean of four experiments.

Enzyme	Total activity in fractions (%)*					Specific activity in fractions (nmoles/min per mg protein)				
	S_m	L_mI	L_mII	H_m	Recovery	S_m	L_mI	L_mII	H_m	Total**
Amine oxidase	9.9	23.6	40.0	22.2	95.7	2.9	46.8	69.8	10.8	15.5
Malate dehydrogenase	69.2	3.6	1.1	21.6	95.5	2271	845	220	1271	1635
Cytochrome *c* oxidase	8.1	5.9	9.6	71.4	95.0	255	1312	1840	3900	1690
Adenylate kinase	68.6	9.4	5.8	3.6	87.4	48.5	45.7	25.0	4.45	37.8
Protein	50.1	7.3	8.4	28.2	94.0					

* 100 % represents the values of the mechanically disrupted mitochondria prior to gradient centrifugation.

** Mechanically disrupted mitochondria prior to gradient centrifugation.

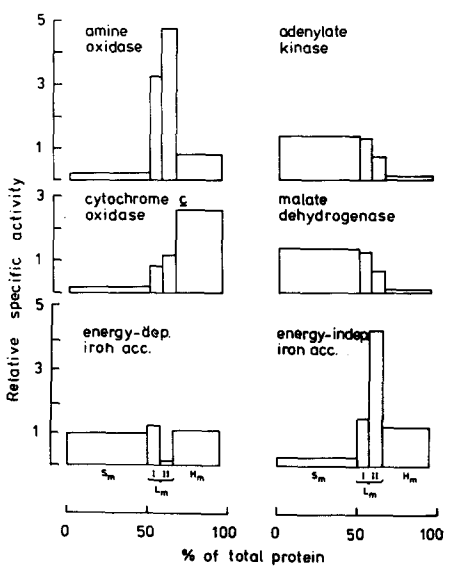

Fig. 1. Distribution of iron in mitochondrial subfractions of rat liver mitochondria in relation to marker enzymes. The mitochondrial subfractions (for symbols, see Table I) were obtained by the combined swelling–shrinking and sonication procedure followed by discontinuous density gradient centrifugation. Abcissa: the percentage of the total protein content in each subfraction presented as cumulative values. Ordinate: the relative specific activity (on a protein basis) of the various fractions taking the specific activity of the iron loaded and mechanically disrupted mitochondria as 1. For experimental details, see Materials and Methods section. The experimental values represent the mean of four different experiments. Recoveries as given in Tables I and II.

TABLE II

SUBMITOCHONDRIAL LOCALIZATION OF IRON IN IRON-LOADED RAT LIVER MITOCHONDRIA

For experimental details and symbols, see Materials and Methods and Table I. The results represent the mean of four experiments.

Fraction	Energy-dependent iron accumulation		Energy-independent iron accumulation	
	Total activity* (%)	Specific activity (nmoles Fe/mg protein)	Total activity* (%)	Specific activity (nmoles Fe/mg protein)
S_m	49.9	5.4	13.9	8.6
L_mI	9.0	6.7	11.0	46.9
L_mII	0.9	0.6	35.5	131.5
H_m	30.7	5.9	34.4	38.0
Swollen, contracted and sonicated mitochondria	100	5.8	100	33.0
Recovery	90.6		94.8	

* 100 % represents the values of mechanically disrupted mitochondria prior to density gradient centrifugation.

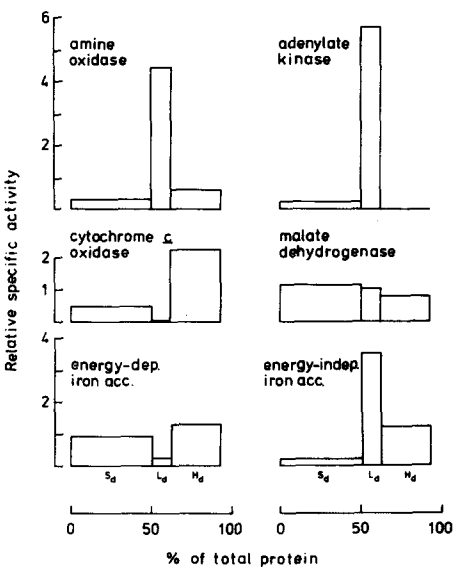

Fig. 2. Distribution of iron in mitochondrial subfractions of rat liver mitochondria in relation to marker enzymes. The mitochondrial subfractions were obtained by the digitonin–Lubrol WX procedure. L_d, light fraction (outer membrane+intermembrane space), S_d soluble fraction (matrix) and H_d, heavy fraction (inner membrane). Abscissa: percentage of total protein content in each subfraction presented as cumulative values. Ordinate: relative specific activity (on a protein basis) of the various fractions taking the specific activity of the iron loaded and digitonin-treated mitochondria as 1. Experimental details as described in Materials and Methods. The figures represent the mean of two experiments. Mean recoveries; protein 93.0 %, amine oxidase 86.2 %, adenylate kinase 81.6 %, cytochrome *c* oxidase 92.3 %, malate dehydrogenase 94.6 %, energy-dependent iron accumulation 88.6 %, energy-independent iron accumulation 91.0 %.

observed as a faint band at the interphase suspension medium–0.75 M sucrose, has only occasionally been mentioned in the literature [18], and in order to exclude insufficient centrifugal effect in our standard procedure, the time integral of $(\text{rev./min})^2$ was increased 2-fold. However, this change did not alter the sedimentation profile whether or not mitochondria were preincubated in the presence of iron.

Distribution of ^{59}Fe following preincubation of mitochondria with $^{59}Fe(III)$–sucrose

From Table II it is seen that approx. 50% of the iron accumulation by an energy-dependent process was recovered in the soluble matrix and intermembrane space, whereas approx. 30% was recovered in the inner membrane. Only 10% of the energy-dependent accumulation could be attributed to the fractions containing outer membrane (L_mI and L_mII). On the other hand, these fractions revealed a significant energy-independent iron accumulation. It should also be noted that the inner membrane appears to accumulate iron (probably as iron(III)–sucrose complex(es) [6]) by an energy-independent process as well.

To exclude the possibility of a significant accumulation of iron in the intermembrane space, the distribution of iron in 'mitoplasts' (inner membrane plus matrix), outer membranes and the intermembrane space was studied (Fig. 2). At a digitonin concentration of approx. 0.2 mg per mg of protein, less than 3% of the energy-dependent and 43% of the energy-independent iron accumulated was recovered in a fraction (L_d) containing 53.5% of the total amine oxidase activity and 69.5% of the total adenylate kinase activity. However, from the amount of protein and the activities of malate dehydrogenase and cytochrome *c* oxidase, this fraction was significantly contaminated with matrix.

Binding of ^{59}Fe to mitochondrial membranes following incubation with $^{59}Fe(III)$–sucrose complex(es)

Mitochondrial outer and inner membranes, prepared by mechanical disruption and density gradient centrifugation, were incubated with $^{59}Fe(III)$–sucrose (0.2 mM of iron) and re-centrifuged on the standard discontinuous density gradient. Again, the outer membranes revealed the highest capacity for the passive binding of iron. The specific activities were; outer membrane, 88.6 nmoles of iron per mg of protein (range 63.2–92.2, $n = 4$) and inner membrane, 32.5 nmoles of iron per mg of protein (range 27.8–55.6, $n = 4$).

DISCUSSION

In previous studies from this laboratory [1, 2, 6–8], rat liver mitochondria were shown to accumulate iron partly by an energy-dependent and partly by an energy-independent mechanism. Furthermore, the mitochondria were shown to possess two classes of iron binding sites with high and low affinity, respectively, where the high-affinity binding sites were linked to the energy-dependent accumulation of iron [6]. In the present study, evidence is presented that this accumulation represents an energy-dependent matrix as well as inner membrane loading with iron.

Although the standard procedures of isolating mitochondrial subfractions do not give absolutely pure preparations due to cross contamination [14, 18], it is important to reduce structure-dependent losses and redistributions of the con-

stituents of the various compartments, including the iron accumulated. In the present study (Table I) the isolated subfractions were not absolutely pure probably due to insufficient separation of the membranes and the soluble phases by the density gradient centrifugation. On the other hand, the yields of the enzymic activities were all in the range 87–95%, ruling out inactivation to any significant extent. Thus, on average, both the yields and purities of the mitochondrial subfractions compare favorably with those previously reported in the literature [14, 19]. Furthermore, redistribution of the iron is probably of minor quantitative significance. First, essentially similar results were obtained by disrupting the iron-loaded mitochondria by chemical and mechanical means (Figs 1 and 2). Secondly, by incubating isolated outer and inner membranes with iron, these membranes accumulated iron at amounts which are equivalent to those of intact mitochondria. Finally, less than 2% of the iron thus accumulated precipitated as insoluble hydroxides when the iron-loaded mitochondria were layered on a discontinuous sucrose gradient and centrifuged as described (see Materials and Methods).

From the data presented in Figs 1 and 2 it is unlikely that the mitochondrial compartments containing amine oxidase and adenylate kinase activities are involved in the energy-dependent accumulation of iron since 80% of the iron thus accumulated was recovered in the soluble matrix (approx. 50%) and inner membrane fractions (approx. 30%).

As far as divalent cations are concerned, the energy-dependent accumulation of Ca^{2+} [20, 21], Sr^{2+} [22] and Ba^{2+} [23] takes place as granules located in the mitochondrial matrix, and these granules have been shown to be cation–phosphate complexes [20–24]. On the other hand, in the present study it is shown that iron cannot be accumulated in the matrix as insoluble phosphate complexes, but in a highly soluble and stable form. As to the nature of the binding sites and storage form of iron in the different mitochondrial compartments, no conclusive experimental data are yet available. However, from studies on the structure of the outer membrane [25], the proposed mechanism of action of digitonin [17], the reactivity of iron with serine derivates [26, 27] and the increased resistance of iron-loaded mitochondria to digitonin, it would be tempting to suggest that iron somehow reacts with cholesterol and/or phosphatidylserine molecules in the outer membrane, making this structure less susceptible to disintegration by digitonin. The molecular events underlying the further binding and transport of iron to the inner compartments of the mitochondria, are not yet known. From studies with rat liver cytosol phosphoproteins, Moret and co-workers [27, 28] have suggested that phosphoproteins may act as iron carriers, at least to the level of the inner membrane. On the other hand, from studies on the structure and distribution of the mitochondrial Ca^{2+}-binding glycoproteins [29, 30], and the similarities between the accumulation of Ca^{2+} and Fe^{3+} [6], the roles played by cytosolic and mitochondrial components in the iron accumulation process are far from settled.

ACKNOWLEDGEMENTS

This study was supported in part by the Norwegian Research Council for Science and the Humanities. The technical assistance of Mrs T. Maröy is greatly acknowledged.

REFERENCES

1 Romslo, I. and Flatmark, T. (1972) Abstr. Commun. Meet. Fed. Eur. Biochem. Soc. 8. No. 639
2 Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 305, 29–40
3 Lehninger, A. L. and Carafoli, E. (1970) in Biochemistry of the Phagocytic Process (Schultz, J., ed.), pp. 9–22, North-Holland Publ. Co., Amsterdam
4 Lehninger, A. L. and Carafoli, E. (1971) Arch. Biochem. Biophys. 143, 506–515
5 Rossi, C. S., Vasington, F. D., Tiozzo, R. and Carafoli, E. (1973) in Mechanisms in Bioenergetics (Azzone, G. F., Ernst, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds.), pp. 157–166, Academic Press, New York
6 Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 325, 38–46
7 Flatmark, T. and Romslo, I. (1973) Abstr. Commun. Int. Congr. Biochem. 9, p. 244
8 Romslo, I. and Flatmark, T. (1973) Abstr. Commun. Int. Congr. Biochem. 9, p. 234
9 Wojtczak, L. and Sottocasa, G. L. (1972) J. Membrane Biol. 7, 313–324
10 Chan, T. L., Greenawalt, J. W. and Pedersen, P. L. (1970) J. Cell. Biol. 45, 291–305
11 Aas, M. (1971) Biochim. Biophys. Acta 231, 32–42
12 Yonetani, T. and Ray, G. S. (1965) J. Biol. Chem. 240, 3392–3398
13 Bergmeyer, H. U. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 757–760, 2nd edn, Verlag Chemie, Weinheim
14 Schnaitman, C. and Greenawalt, J. W. (1968) J. Cell. Biol. 38, 158–175
15 Flatmark, T., Terland, O. and Helle, K. B. (1971) Biochim. Biophys. Acta 226, 9–19
16 Hinton, R. H., Burge, M. L. E. and Hartman, G. C. (1969) Anal. Biochem. 29, 248–256
17 Schnaitman, C., Erwin, G. V. and Greenawalt, J. W. (1967) J. Cell. Biol. 32, 719–735
18 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438
19 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) in Methods of Enzymology (Eastbrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 448–463, Academic Press, New York
20 Greenawalt, J. W., Rossi, C. S. and Lehninger, A. L. (1964) J. Cell Biol. 23, 21–38
21 Brierley, G. P. and Slautterback, D. B. (1964) Biochim. Biophys. Acta 82, 183–186
22 Greenawalt, J. W. and Carafoli, E. (1966) J. Cell Biol. 29, 37–61
23 Peachey, L. D. (1964) J. Cell Biol. 20, 95–111
24 Weinbach, E. C. and von Brand, T. (1965) Biochem. Biophys. Res. Commun. 19, 133–137
25 Ernster, L. and Kuylenstierna, B. (1970) in Membranes of Mitochondria and Chloroplasts (Racker, E., ed.), pp. 172–193 van Nostrand Reinhold Co., New York
26 Wang, C. C. and Newton, A. (1969) J. Bacteriol. 98, 1143–1150
27 Donella, A., Pinna, L. A. and Moret, V. (1972) FEBS Lett. 26, 249–251
28 Pinna, L. A., Clari, G. and Moret, V. (1971) Biochim. Biophys. Acta 236, 270–278
29 Carafoli, E., Gazzotti, P., Saltini, C., Rossi, C. S., Sottocasa, G. L., Sandro, G., Panfili, E. and de Bernard, B. (1973) in Mechanisms in Bioenergetics (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds), pp. 293–307, Academic Press, New York
30 Carafoli, E. (1973) Abstr. Commun. Int. Congr. Biochem. 9, p. 255