SUBMITOCHONDRIAL LOCALIZATION OF TRANSFERRIN AND IRON ACCUMULATED BY ISOLATED RAT LIVER MITOCHONDRIA

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1. Introduction

The mechanisms whereby iron is taken up by eucaryotic cells and incorporated into protoporphyrin are only partially explained. The final insertion of iron into the protoporphyrin is catalyzed by ferrochelatase which is tightly bound to the matrix side of the mitochondrial inner membrane [1]. To reach this mitochondrial compartment iron has to pass the outer as well as the inner mitochondrial membrane. It has been suggested that transferrin molecules which bind to the surface membrane receptor are subsequently taken inside the cell in the process of endocytosis involving microtubular like proteins [2–7]. Further stages of the mechanism by which transferrin releases its iron at sites on the mitochondria are not yet completely elucidated. For dissociation of the transferrin–iron complex protons must be donated to transferrin and iron must be reduced by a mechanism that depends on an intact respiratory chain in mitochondria [8,9]. Moreover, there exists a coordinated mechanism for the release of HCO_3^- and iron from transferrin [10]. The enzymic removal of HCO_3^- from transferrin could result in the release of iron [11,12].

The iron–transferrin complex has been used as the donor compound and it has been shown that isolated rat liver mitochondria accumulate iron from transferrin. The processes are found to have saturation kinetics, temperature dependency, a pH optimum as well as an energy requirement [13]. The uptake of iron was inhibited by haemin and stimulated by isonicotinic acid hydrazide [14], as well as markedly influenced by chelating agents and phosphate compounds [15].

The present paper deals with the submitochondrial localization of transferrin and iron accumulated by isolated rat liver mitochondria.

2. Materials and methods

Preparation and purification of mitochondria and iron accumulation experiments were performed as in [15].

2.1. *Preparation of [^{59}Fe]transferrin and ^{125}I-labelled transferrin*

[^{59}Fe]Transferrin was prepared as in [4]. To remove unbound ^{59}Fe, the iron–transferrin solution was dialyzed for 24 h against 20 mM $NaHCO_3$. The iodination procedure was based on that in [16]. $K^{125}I$ was added to transferrin in 50 mM sodium phosphate buffer (pH 7.5) at 10 mg/ml in an ice bath. The iodination procedure was initiated by the addition of chloramine-B and after 10 min the reaction was terminated by sodium metabisulphite. To remove unbound ^{125}I, ^{125}I-labelled transferrin was passed though a Sephadex G-100 column, equilibrated with 50 mM sodium phosphate buffer (pH 7.5).

2.2. *Fractionation of mitochondria*

Mitoplasts (inner membranes plus matrix) were obtained by the method in [17] using 0.16–0.18 mg digitonin/mg mitochondrial protein or the procedure in [18] was applied. In the latter the pellet of iron-loaded mitochondria was suspended in 10 mM phosphate buffer (pH 7.4) for 10 min at 0°C. Subsequently, 2.26 M sucrose was added. After 10 min, the ice-cold suspension was sonicated 10 s in an

MSE ultrasonic disintegrator and thereafter diluted to obtain 260 mM sucrose. Finally, it was layered on top of a discontinuous density gradient of sucrose (12 ml 1.17 M and 12 ml 0.76 M sucrose) and centrifugated at 90 000 × *g* for 3 h in a L5-65 Beckman ultracentrifuge, rotor SW 27.1, 6 × 38 ml. The soluble intermembrane space and matrix constituents were found in the input layer (S). Outer membranes partially contaminated by matrix constituents were recovered in the interphase 0.75–1.17 M sucrose (L). Inner membranes were recovered at the bottom of the tube (H). The fractions were aspirated and diluted with appropriate volumes of the incubation medium for counting of radioactivity, enzymic assays and protein determination.

2.3. *Enzyme assays*

In purified mitochondria and their fragments the activities of the following enzymes were estimated: (1) succinate dehydrogenase (EC 1.3.9.9) according to [19]; (2) monoamine oxidase (EC 1.4.3.4) by means of our modification of the spectrophotometric method [20]; (3) rotenone insensitive NADH-cytochrome *c* reductase according to [21]; (4) aconitase (EC 4.2.1.3) by means of the modified method [22]. Assays for enzymic activities were performed in triplicate and the average values are given. Protein was estimated by means of the biuret test. Iron was estimated by the method in [23]. Activities of enzymes were expressed in % of the sum of activities in the subfractions and as specific activities (spec. act.) in nmol.min^{-1} .mg protein^{-1}.

3. Results

3.1. *Isolation of submitochondrial fractions*

This was by the method in [17]. Total and specific activities of the chosen enzymes in both mitochondrial subfractions, obtained by the incubation with digitonin, are shown in table 1. In the control experiments 70% of the NADH-cytochrome *c* reductase activities and 41% of the monoamine oxidase activities were recovered in the supernatant (outer membranes and the intermembrane space) whereas 80% of the succinate dehydrogenase activity was found in the mitoplast fraction. When iron-loaded mitochondria were fractionated only ~25% of the NADH-cytochrome *c* reductase activities and ~15% of the monoamine oxidase activities were recovered in the supernatant. The distribution pattern of succinate dehydrogenase does not undergo any

Table 1
Distribution of iron and transferrin in mitochondrial subfraction of rat liver mitochondria in relation to marker enzymes

Fraction	Succinate dehydrogenase		Monoamine oxidase		NADH-cytochrome *c* reductase		Radioactivity	
	Spec. act.	%[a]	Spec. act.	%	Spec. act.	%	Spec. act.[b]	%
Control mitochondria	62.7		5.0		32.2			
Outer membrane + intermembrane space	26.0	20	4.2	41	46.8	70		
Mitoplasts	93.4	80	5.5	59	18.0	30		
^{59}Fe labelled mitochondria	51.4		5.6		36.8		94.1 (946)	
Outer membrane + intermembrane space	20.6	21	1.9	16	8.5	24	49.8 (491)	25
Mitoplasts	70.1	79	8.9	84	24.0	76	134.0 (1321)	75
^{125}I-labelled mitochondria	46.5		5.3		32.2		5.3 (294)	
Outer membrane + intermembrane space	16.7	17	1.4	13	6.4	26	7.6 (412)	70
Mitoplasts	77.7	83	9.8	87	17.9	74	3.2 (172)	30

[a] 100% represents the sum of enzyme activities or radioactivities in both submitochondrial fractions
[b] pmol iron.mg protein^{-1} or pmol transferrin.mg protein^{-1} and in parentheses cpm.min^{-1}.mg protein^{-1}

changes. Only 25% of the ^{59}Fe radioactivity could be attributed to the fraction containing outer membrane and the intermembrane space. On the other hand, this fraction revealed 70% of the ^{125}I radioactivity. It should be noted that there is an important difference in the amount of accumulated iron and transferrin. The uptake of iron was markedly influenced by chelating agents and phosphate compounds [15], while the accumulation of ^{125}I-labelled transferrin was not influenced by these compounds (fig.2).

3.2. *Isolation of mitochondrial subfragments*

This was by the method in [18]. The distribution of the activities of marker enzymes and iron in mitochondrial subfractions obtained by mechanical disruption are shown in fig.1. A low percentage of enzymes of mitochondrial membranes is observed in fraction S which seems to be a rather pure preparation of the soluble intermembrane space and matrix constituents. Only 17% of iron accumulated could be found in fraction S. In fraction L there is ~50% of monoamine oxidase, NADH-cytochrome *c* reductase and aconitase activities and only 17% of the succinate dehydrogenase activity. Thus, fraction L represents outer membranes contaminated by matrix constituents and 28% of the iron was recovered in it. The distribution of enzymic activities in fraction H suggests that it mainly contains inner membranes (80% of the succinate dehydrogenase activity) contaminated by outer membranes. This fraction reveals a significant iron accumulation (55%).

If the pellet of iron-loaded mitochondria was washed in the presence of 1.25 mM EDTA, ~25% of the iron was removed during centrifugation. EDTA which forms stable complexes with iron is unable to cross the inner mitochondrial membrane [24]. Therefore, iron which was washed out with EDTA during centrifugation was presumably released from the outer mitochondrial compartment. Comparing the results obtained from the fractionation (fig.1) it appears that some part of iron which is bound to the outer membrane can be chelated by EDTA, but most of the iron is situated inside the inner mitochondrial compartment and is therefore not accessible to EDTA.

3.3. *Uptake of iron and transferrin by the mitoplasts*

It was found that the mitoplasts accumulated iron and transferrin by mechanisms in many ways similar to the uptake of iron and transferrin for mitochondria (fig.2,3).

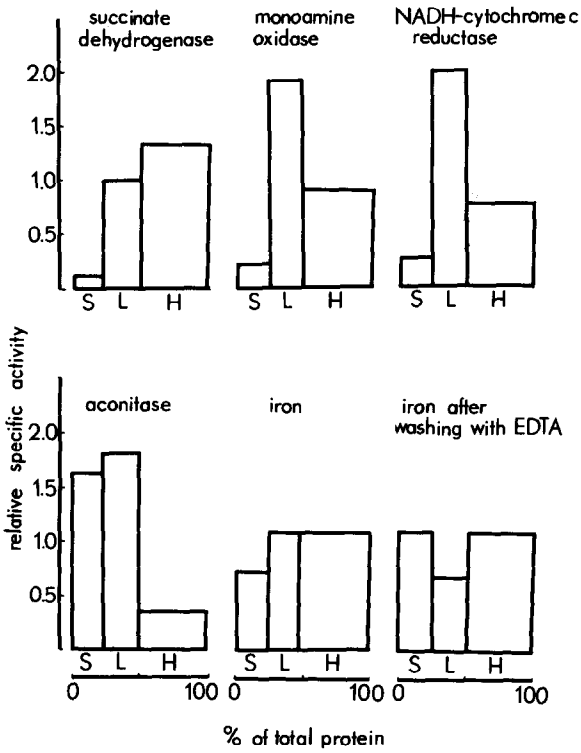

Fig.1. Distribution of iron in mitochondrial subfractions of rat liver mitochondria in relation to marker enzymes. Abcissa: the percentage of the total protein content in each subfraction. Ordinate: the relative specific activity of the various fractions taking the specific activity of the iron loaded mitochondria as 1. The final concentration of iron and transferrin in the reaction mixture as 0.96 μM and 0.6 μM, respectively. Protein concentration was 2.0 mg/ml. ATP concentration was 2.5 mM.

4. Discussion

The results obtained here support the observation, that the dissociation of iron–transferrin complex takes place during the uptake of iron by the mitochondria. The amount of ^{125}I-labelled transferrin bound with mitochondria is low and its accumulation is influenced neither by chelating agents nor phosphate compounds. The iron-loaded mitochondria prove to have an increased resistance to digitonin, which

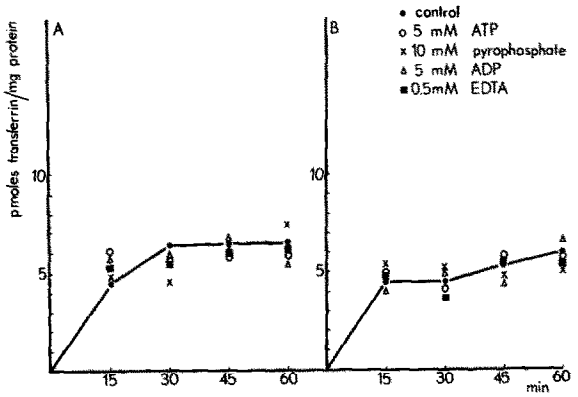

Fig.2. The uptake of ^{125}I-labelled transferrin by mitochondria (A) and mitoplasts (B) as a function of time. The final concentrations of iron and transferrin in the reaction mixture were 1.78 μM and 1.13 μM, respectively. Protein concentration was 1.74 mg/ml.

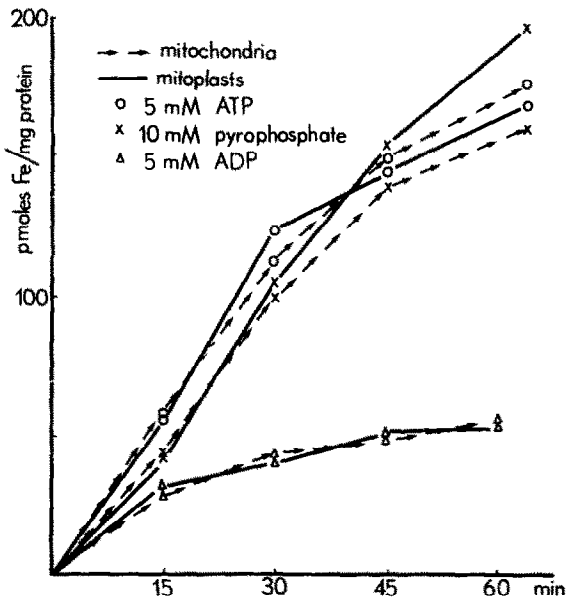

Fig.3. The uptake of iron by mitochondria and mitoplasts as a function of time. Experimental conditions as in fig.2.

remains in agreement with the results [25]. Despite that, ~70% of ^{125}I-label radioactivity is separated together with the outer membrane fraction after treatment with digitonin. It can be assumed, that the little amount of transferrin which is incorporated in mitochondria is mainly bound with outer membranes. The increased resistance to digitonin does not allow determination of the localization of the accumulated iron. In connection with this the other method of fractionation, i.e., the mechanical disruption was applied. It can be concluded, that iron which is accumulated by the isolated mitochondria from the iron–transferrin complex reaches the inner mitochondrial compartment and is mainly bound with inner membranes. Only 25% of iron which was chelated by EDTA is situated inside the outer mitochondrial compartment.

The partial removal of outer membranes under digitonin treatment does not influence the incorporation of iron and transferrin by the fraction of mitoplasts, neither does it change the influence of chelating agents and phosphate compounds on this process. However, the received fraction of mitoplasts contains a great percentage of the activities of enzymes of outer membranes. With the use of the iron–sucrose complex as a donor compound, the fractions of outer membranes have revealed a significant energy-independent iron accumulation [25] and it cannot be excluded that dissociation of the iron–transferrin complex occurs inside the outer mitochondrial compartment. Probably, this stage of the process is influenced by the chelating agents and phosphate compounds.

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