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ENERGY-DEPENDENT ACCUMULATION OF IRON BY ISOLATED RAT LIVER MITOCHONDRIA

II. RELATIONSHIP TO THE ACTIVE TRANSPORT OF Ca^{2+}

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SUMMARY

1. Rat liver mitochondria isolated in sucrose–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid medium possess high- and low-affinity binding sites of iron when $^{59}Fe(III)$–sucrose is used as a soluble and stable model complex. At pH 7.4 the low-affinity binding sites bind about 60 nmoles of iron per mg of protein with $K_m' \approx 0.4$ mM. The high-affinity sites bind about 10 nmoles of iron per mg of protein with $K_m' \approx 30$ μM and is completely inhibited by the uncoupler carbonyl cyanide-*m*-chlorophenylhydrazone.

2. When the mitochondria were incubated with Fe(III)–[^{14}C]sucrose as the substrate, the complex is accumulated by an energy-independent mechanism. Thus, the energy-independent binding of iron is largely due to binding of the Fe(III)–sucrose complex(es).

3. The energy-dependent iron accumulation is inhibited by La^{3+}, ruthenium red, hexaminecobalt(III) chloride and sulphydryl reagents, *i.e.* by reagents which all inhibit the energy-dependent Ca^{2+} uptake. Furthermore, Ca^{2+} inhibits the energy-dependent accumulation of iron in a competitive manner ($K_i' = 115$ μM) without affecting the energy-independent accumulation. These results suggest that the mechanism of the energy-dependent loading with iron is similar to that of Ca^{2+}.

INTRODUCTION

It has been known for some time that mitochondria from various sources bind iron in the absence of electron transport[1,2], but only recently evidence was presented for the presence of an energy-linked accumulation of iron by rat liver mitochondria[3,4]. Thus, by using $^{59}Fe(III)$–sucrose as a soluble and stable model complex, rat liver mitochondria were found to accumulate iron from the suspending medium partly by an energy-independent and partly by an energy-dependent process, both of which showed different time, pH and temperature dependencies.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; PHMB, *p*-hydroxymercuribenzoate, TMPD, *N*, *N*, *N'*, *N'*,-tetramethyl-*p*-phenylenediamine.

In this respect, the iron accumulation revealed features in common with the mitochondrial accumulation of Ca^{2+} (ref. 5).

Rat liver mitochondria have been shown to possess two classes of Ca^{2+} binding sites[5]. One class, large in number, has a relatively low affinity for Ca^{2+} and corresponds to the respiration-independent Ca^{2+} binding[6,7]. The other has an extremely high affinity for Ca^{2+}, but is present in a small number and proposed to be involved in the energy-linked Ca^{2+} accumulation[5]. The high-affinity Ca^{2+} binding is inhibited by uncouplers[5], lanthanides[8] and ruthenium red[9]. Divalent cations (Sr^{2+}, Mn^{2+} and Zn^{2+}) compete with Ca^{2+} for the high-affinity binding sites[5] which are thought to be represented by glycoprotein(s) partitioned between the various mitochondrial compartments[10,11].

In the present paper certain properties of the two iron-binding processes[3,4] will be described in more detail. It is concluded that the energy-dependent iron accumulation has many features in common with that of Ca^{2+}.

MATERIALS AND METHODS

Animals and preparation of mitochondria

Rat liver mitochondria were prepared essentially as previously described[4], except that the isolation medium consisted of 0.25 M sucrose and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 7.4. The functional integrity of the preparations was tested by measuring the respiratory control ratio with ADP (R. $C._{ADP}$)[12] using succinate as the substrate. Only mitochondria with R. $C._{ADP}$ values >2.5 were used.

Preparation of ^{59}Fe(III)–sucrose and Fe(III)–[^{14}C]sucrose

Soluble and stable complexes of ^{59}Fe(III)–sucrose and Fe(III)–[^{14}C]sucrose were prepared as described[4]; the products thus obtained contained approx. 100000 cpm/μmole of iron and approx. 15000 cpm/μmole of sucrose, respectively.

Accumulation of iron and sucrose

The mitochondria were incubated at 25 °C essentially as described[4] in a medium containing in a volume of 3.0 ml: 225 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl and 10 mM HEPES buffer, pH 7.4. Following a preincubation period of 10 min either ^{59}Fe(III)–sucrose, Fe(III)–[^{14}C]sucrose or [^{14}C]sucrose was added and the reaction was allowed to proceed for 30 s. Aliquots of 1 ml were then withdrawn, transferred to the precooled (0–2 °C) discontinuous density gradient, containing 20 mM EDTA, and further processed as described[4].

Spectrophotometry

The measurement of oxidation–reduction level of the mitochondrial *b*-type cytochromes were performed as previously described[13].

Protein was determined using the Folin-Ciocalteau reagent[14].

Chemicals

Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), *N*-ethylmaleimide, *p*-hydroxymercuribenzoate (PHMB) and ruthenium red were obtained from the

Sigma Chemical Co. (St Louis, Mo., U.S.A.). HEPES (A grade) was purchased from Calbiochem (Luzern, Switzerland), $LaCl_3$ from Koch-Light Laboratories Ltd (Colnbrook, England), [^{14}C]sucrose from The Radiochemical Centre, Amersham, England, and $^{59}FeCl_3$ from Institutt for Atomenergi, Kjeller, Norway. Hexaminecobalt(III) chloride was prepared as described by Glemser[15]. Other chemicals were of highest purity commercially available.

RESULTS

Low-affinity and high-affinity binding sites of iron

Rat liver mitochondria were examined for their capacity to accumulate iron in State 1 respiration by determining the amount of iron accumulated on adding increasing concentrations of ^{59}Fe(III)–sucrose in the absence as well as in the presence of uncoupler (CCCP). State 1 respiration was selected due to the high association constants of iron with di- and tricarboxylic acids[16]. Under the given experimental conditions, the energy-dependent iron accumulation represented approx. 40% of the total. By plotting the data in a Scatchard plot a biphasic curve was obtained (Fig. 1) where the horizontal rectilinear leg corresponds to low-affinity and the vertical leg to high-affinity binding sites. The apparent number of binding sites, as obtained from the extrapolated intercepts[5], was $n'_l = 60.1$ nmoles per mg of protein and $n'_h = 10.6$ nmoles per mg of protein for the low- and high-affinity binding, respectively. In the presence of CCCP the Scatchard plot was monophasic, with a slope corresponding to that of the low-affinity leg of the biphasic curve in the absence of uncoupler. A similar plot of the energy-dependent iron accumulation yielded a monophasic curve; the extrapolated intercept gave a value of $n'_h = 8.1$ nmoles per mg of protein (figure not shown).

The nature of the high- and low-affinity binding sites was further explored by incubating the mitochondria with Fe(III)–[^{14}C]sucrose (Fig. 2). In contrast to the results with ^{59}Fe(III)–sucrose, CCCP had no effect on the binding of Fe(III)–[^{14}C]sucrose. From the difference in the accumulation of [^{14}C]sucrose and its

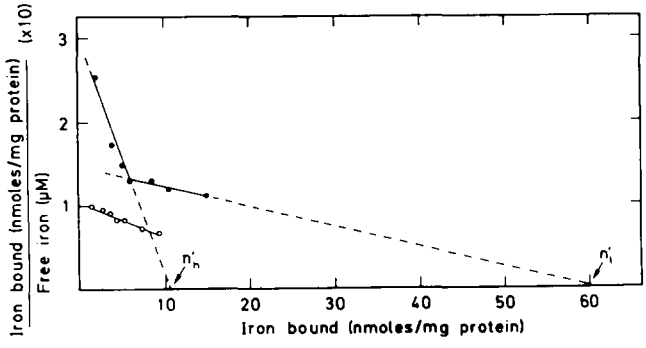

Fig. 1. Effect of Fe(III)–sucrose concentration on the iron accumulation in rat liver mitochondria (Scatchard plots). The mitochondria were suspended in the standard incubation medium (see Methods) at a protein concentration of 1.8–3.4 mg/ml. The incubation period was 30 s; 25 °C. ●, total iron accumulation; ○, iron accumulation in the presence of 17 μM CCCP. The mitochondria were preincubated for 10 min with the uncoupler before the FeIII–sucrose was added. All points represent the mean of four different experiments.

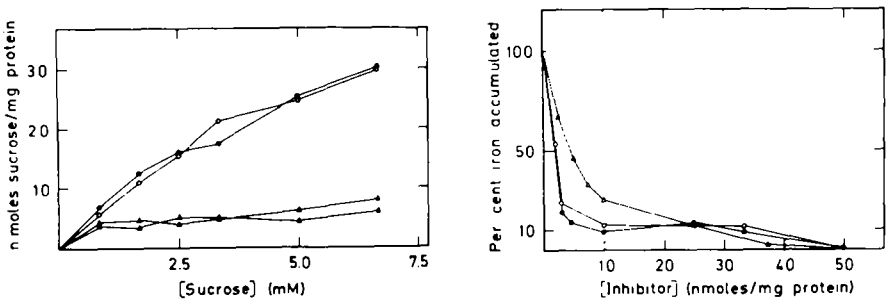

Fig. 2. Effect of Fe^{3+}–[^{14}C]sucrose and [^{14}C]sucrose concentration on the accumulation of radioactive sucrose in rat liver mitochondria. Incubation conditions as in Fig. 1; for details, see Methods. Accumulation of Fe^{3+}–sucrose (● and ○) and sucrose (▲ and △) in the absence (closed symbols) and presence (open symbols) of 17 μM CCCP. All points represent the mean of two different experiments.

Fig. 3. Inhibition of the energy-dependent accumulation of iron by La^{3+} (●), ruthenium red (○) and hexaminecobalt (III) chloride (△). Experimental conditions as in Fig. 1 except that a fixed concentration of iron (0.25 mM) was used and added simultaneously with the inhibitors. The 100% values were 9.8 nmoles/mg protein (●), 10.2 nmoles/mg protein (○) and 9.2 nmoles/mg protein (△), respectively.

iron complex(es) a binding of the Fe(III)–sucrose complex(es) to the mitochondrial membranes is clearly demonstrated.

Effect of ruthenium red and hexaminecobalt(III) chloride

The discovery of Moore[9] that the mucopolysaccharide stain, ruthenium red, inhibited the high-affinity, energy-dependent accumulation of Ca^{2+} in mitochondria started a number of studies on divalent cation transport phenomena in the presence of this complex cation[18–20]. Recently, another complex cation, hexaminecobalt(III), which is known to react with negatively-charged groups of sulphate-containing glycosaminoglycanes[21] has been shown to be an equally potent inhibitor of the energized Ca^{2+} translocation without affecting the mitochondrial energy state[22]. From their effect on the energy-linked uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} it was of great interest to study the effect of ruthenium red and hexaminecobalt(III) chloride on the mitochondrial accumulation of iron.

From Fig. 3 it is seen that ruthenium red almost completely blocked the energy-dependent iron accumulation at a concentration of only 10 nmoles per mg of protein; 50% inhibition was obtained at approx. 2.5 nmoles per mg of protein. The influence on the energy-independent iron accumulation was negligible at these low concentrations. On the other hand, at concentrations above 25 nmoles per mg of protein, the energy-independent accumulation was largely increased by ruthenium red (figure not shown). However, at these high concentrations, the mitochondria revealed a tendency to aggregate.

Similarly, it is seen from Fig. 3 that hexaminecobalt(III) chloride also effectively inhibited the energy-dependent accumulation of iron; 50% inhibition was obtained at approx. 4.3 nmoles per mg of protein. On the other hand, at all concentrations of hexaminecobalt(III) chloride the energy-independent accumulation was largely increased.

Effect of La^{3+}

From Fig. 3 it is seen that La^{3+} at a concentration of 2.5 nmoles per mg of protein inhibited the energy-dependent accumulation of iron by about 50%. On the other hand, La^{3+} slightly increased the energy-independent iron accumulation.

Effect of sulphydryl reagents

At concentrations of 20–30 nmoles per mg of protein, the sulphydryl reagents *N*-ethylmaleimide and PHMB inhibited the energy-dependent iron accumulation by 60 and 90%, respectively, without affecting the energy-independent accumulation (Figs 4A and 4C). However, at concentrations above 50 nmoles per mg of protein, PHMB markedly inhibited the uncoupler-insensitive accumulation.

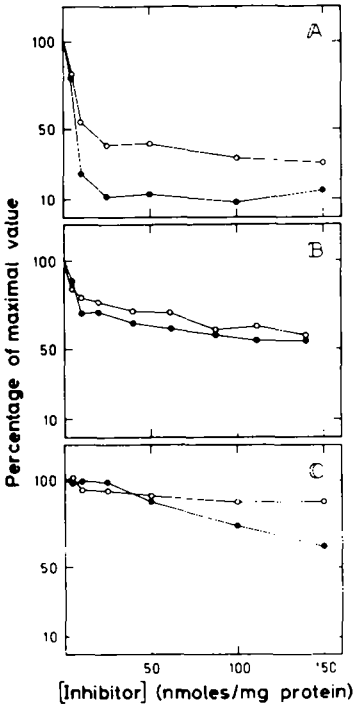

Fig. 4. (A and C) Inhibition of the energy-dependent (A) and energy-independent (C) accumulation of iron by PHMB (●) and *N*-ethylmaleimide (○). Experimental conditions as in Fig. 1 except that a fixed concentration of iron (0.25 mM) was used. The mitochondria were preincubated for 10 min with the sulphydryl reagents. The 100% values were 6.7 nmoles/mg protein (A) and 18.7 nmoles/mg protein (C). (B) The effect of PHMB (●) and *N*-ethylmaleimide (○) on the extent of CCCP-induced oxidation of the cytochrome *b* complex. The mitochondria were suspended in the standard incubation medium (see Methods) at a protein concentration of 6.0 mg per ml and supplemented with 3.3 mM KCN, 3 μM rotenone, 4.2 mM ascorbate and 90 μM TMPD. 10 μM CCCP were added to induce complete deenergization. 100% oxidation was taken as the difference in transmission between the level obtained by adding CCCP and the initial ascorbate/TMPD level. The mitochondria were preincubated for 10 min with the sulphydryl reagents.

*Effect of various inhibitors on the mitochondrial energy potential**

Recently it has been shown that the redox state of the cytochrome *b* complex of the respiratory chain is dependent on the phosphate potential[13,17,23-28], and this makes it suitable as an internal probe of the energy state of the mitochondrial inner membrane[13,17,27]. Thus, this parameter has been shown to be very useful to study factors affecting the generation and dissipation of the energy potential[13,17,27], and in the present study the effect of the various inhibitors on the energy-dependent accumulation of iron has been tested.

By using the uncoupler-induced oxidation of the cytochrome *b* complex as a quantitative measure of the mitochondrial energy potential (for details, see ref. 13), it is seen from Table I that preincubation of the mitochondria with La^{3+} reduced the potential approx. 10% whereas ruthenium red had no effect. On the other hand, the sulphydryl reagents (PHMB and *N*-ethylmaleimide) both markedly lowered the mitochondrial energy potential (Fig. 4B).

TABLE I

EFFECT OF RUTHENIUM RED, HEXAMINECOBALT(III) CHLORIDE AND La^{3+} ON THE CCCP-INDUCED OXIDATION OF THE CYTOCHROME *b* COMPLEX

The mitochondria were suspended in the standard incubation medium, pH 7.40, at 6.0 mg of protein per ml in the presence of 50 μM ruthenium red, hexaminecobalt (III) chloride or La^{3+}. Experimental procedure as described in Fig. 4B.

Compound	*CCCP-induced oxidation* [$\triangle$T(%)]		*Per cent of control*
	Mean	*Range*	
Control	0.424	0.380–0.532 ($n=6$)	100
Ruthenium red	0.438	0.392–0.492 ($n=3$)	103.2
Hexaminecobalt (III) chloride	0.396	0.344–0.432 ($n=3$)	93.5
La^{3+}	0.380	0.354–0.404 ($n=3$)	89.6

Competition between iron and Ca^{2+}

Whereas Ca^{2+} had no effect on the iron accumulation in uncoupled mitochondria (Fig. 5B), the energy-dependent accumulation of iron was markedly inhibited by Ca^{2+} with $K'_i = 115$ μM (Fig. 5A).

DISCUSSION

In a recent study from this laboratory[4] it was shown that rat liver mitochondria largely increase their content of iron when incubated with Fe(III)–sucrose as a soluble and stable iron complex. The accumulation proceeds by two different mechanisms, *i.e.* by an energy-independent as well as by an energy-dependent mechanism. The latter was found to depend on a high energy state of the mitochondrial inner membrane generated by oxidation of endogenous substrates as well as by hydrolysis of endogenous ATP. This conclusion is further supported by the

* The term energy potential is used synonymously with the primarily conserved energy[17].

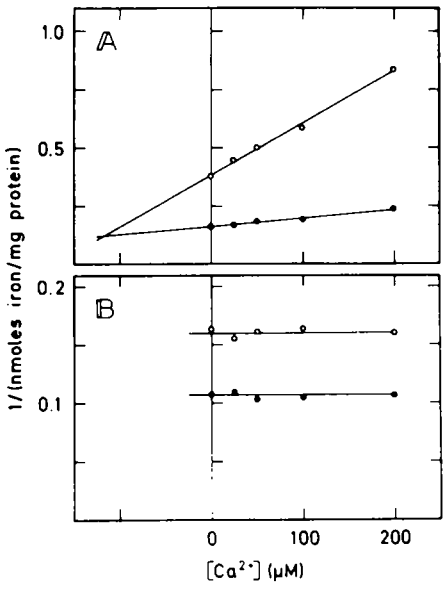

Fig. 5. The effect of Ca^{2+} on the energy-dependent (A) and the energy-independent (B) iron accumulation. Experimental conditions as in Fig. 1 except that fixed concentrations of iron, *i.e.* 250 μM (●) and 83 μM (○) were used and added simultaneously with Ca^{2+}.

present study where a biphasic Scatchard plot (Fig. 1) indicates that rat liver mitochondria contain two classes of binding sites for iron and/or its sucrose complex[29]. One class, large in number has a relatively low affinity and is undoubtedly similar to the energy-independent iron accumulation previously described[4]. The other class with a higher affinity is present in a smaller number. The high-affinity binding sites are completely inhibited by uncoupler suggesting that these sites are involved in the energy-dependent iron accumulation.

Several lines of evidence point to a difference in the mechanism of the energy-independent binding of iron and Ca^{2+}. First, the iron binding decreases with increasing pH whereas the Ca^{2+} binding increases[4]. Secondly, studies with an Fe(III)–[^{14}C]sucrose complex (Fig. 2) suggest that the low-affinity iron binding mainly represents the binding of the complex and not of Fe^{3+}. Thirdly, the energy-independent binding of iron is not inhibited by Ca^{2+}. Finally, the effects of ruthenium red and hexaminecobalt(III) chloride on the two processes are different (see below).

On the other hand, the energy-dependent accumulation of iron shares many properties in common with that of Ca^{2+}. Thus, as far as the concentration and energy dependence is concerned, the accumulation of iron is analogous to that of Ca^{2+} (ref. 5). This similarity also holds for the inhibition of the energy-dependent or high-affinity accumulation of both cations by ruthenium red[9,18–20] and hexaminecobalt(III) chloride[22] (Fig. 3). Thus, 50% inhibition of the iron accumulation was obtained at about 2.5 nmoles of ruthenium red per mg of protein, which compares favorably well with its inhibition of the energy-dependent Ca^{2+} uptake[9]. On the other hand, whereas ruthenium red inhibits both high- and low-affinity binding of Ca^{2+} (ref. 19), only energy-dependent binding of iron was inhibited.

Recently Tashmukhamedov *et al.*[22] reported that hexaminecobalt(III) chloride is a powerful inhibitor of Ca^{2+} translocation affecting neither the ADP/O ratio nor the respiratory control with ADP. This result is in agreement with the present study where the energy potential was found to be unaffected by this reagent (Table I). Since hexaminecobalt(III) chloride reacts with compounds similar to those reacting with ruthenium red and neither of them affects the mitochondrial energy state, it is tempting to suggest that both reagents inhibit the energy-dependent iron accumulation by titrating out the high-affinity binding sites.

Another similarity to the Ca^{2+} accumulation is the influence of La^{3+}. However, La^{3+} was found to inhibit the energy-dependent or high-affinity iron binding sites at a higher concentration than necessary to inhibit Ca^{2+} and Mn^{2+} accumulation[5], *i.e.* similar to the inhibition of the energized accumulation of lead[30].

Sulphydryl reagents which are well-known inhibitors of mitochondrial P_i transport are also known to interfere with the energy transduction in the mitochondrial inner membrane[31,32]. In the present study where no P_i was added to the incubation medium (see Methods and ref. 4) it is shown (Fig. 4B) that these reagents induce a decrease in the mitochondrial energy potential. Thus, the inhibition by sulphydryl reagents of the energy-linked iron accumulation could be ascribed to a partial deenergetization of the mitochondrial inner membrane, similar to the inhibition of the energy-linked Ca^{2+} accumulation in inner membrane vesicles[33]. At concentrations <30 nmoles of sulphydryl reagents per mg of protein, the energy-independent iron accumulation was unaffected, in agreement with the findings of Cederbaum and Wainio[34]. However, by titrating out the mitochondrial SH-pool (*i.e.* at 50–100 nmoles sulphydryl reagents per mg of protein[35]) a marked decrease in the uncoupler-insensitive iron binding was observed.

The failing influence of CCCP on the mitochondrial binding of Fe(III)–[^{14}C]sucrose (Fig. 2) reflects (as discussed above) an energy-independent binding of the Fe(III)–sucrose complex(es) to the mitochondrial membranes. Furthermore, the present study has shown that a certain fraction of the complex(es) dissociates and that iron ions are taken up by the mitochondria by an energy-dependent mechanism. Thus, it would be tempting to suggest that whereas the low affinity binding is linked to the energy-independent binding of the Fe(III)–sucrose complex, the high-affinity binding represents an energy-dependent loading with Fe^{3+}. This conclusion is further supported by the selective inhibition of the energy-dependent iron accumulation by La^{3+}, ruthenium red and hexaminecobalt(III) chloride, *i.e.* reagents which all inhibit the energy-dependent membrane and matrix loading with Ca^{2+} (refs 5, 9, 22). From Fig. 5 it is also seen that Ca^{2+} competitively inhibits the energy-dependent iron accumulation, and more recent studies in this laboratory[36,37] also suggest that the energy-dependent loading with iron is similar to that of Ca^{2+}.

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