FERRITIN IRON AS SUBSTRATE FOR SYNTHESIS OF PROTOHEME IN INTACT RAT LIVER MITOCHONDRIA

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1. Introduction

The final step in the biosynthesis of heme takes place within the innermembrane of the mitochondria where the ferrochelatase (EC 4.99.1.1.) catalyzes the insertion of Fe(II) into protoporphyrin IX [1,2]. For this reason the mitochondria are the ultimate destination for a major part of the iron entering the cell [3]. At present little is known about the pathway of iron transport across cytosol and the nature of the compound(s) donating iron to the mitochondria (review [3,4]). In vitro studies have focused particularly on transferrin [5–7], low M_r iron-binding compounds [4,8,9] and ferritin [10,11] as possible iron donors to the mitochondria. We have described a FMN-dependent ferrireductase reaction by means of which isolated mitochondria can mobilize iron from ferritin [12–15]. The mitochondria possess a mechanism for the reduction of exogenous FMN [14], and $FMNH_2$ is used as the obligatory small M_r reductant to release iron from the ferritin molecules [16]. The reaction depends on low concentrations of oxygen to protect $FMNH_2$ against autoxidation [14]. In these studies the mitochondria were incubated either with bathophenanthroline to determine release of iron from ferritin, or with deuteroporphyrin IX as precursor for deuteroheme. However, depending on the concentration, these compounds are potent loose-couplers and swelling agents to the mitochondria [17–19], and these unphysiological effects might interfere with the interaction between ferritin and the mitochondria.

Abbreviations: Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; RC_{ADP}, respiratory control ratio with ADP; M_r, relative molecular mass

Here, it is shown that mitochondria with intact energy coupling and energy conserving properties mobilize iron from ferritin and insert the iron into protoporphyrin IX, the natural precursor of heme. The rate of mobilization of iron from ferritin is ~3-times the rate of formation of protoheme. From a physiological point of view these results are important with respect to a possible role for ferritin as donor of iron to the mitochondria in situ.

2. Materials and methods

Rat liver mitochondria were prepared as in [20]. The functional integrity of the mitochondria was tested by measuring the respiratory control ratio with ADP (RC_{ADP}) using succinate as substrate. The RC_{ADP} of the mitochondria used was in the range 5–8. Native horse spleen ferritin (A grade, Cd^{2+}-free from Calbiochem, Luzern) was fractionated according to its iron content to give fractions of defined iron: protein ratios [13]. Protoporphyrin IX was from Porphyrin Products (Logan UT). Safranine was from Merck AG (Darmstadt). ADP, bathophenanthroline, CCCP, FMN (grade 1) and Hepes were from Sigma Chemical Co. (St Louis MO). Mobilization of iron from ferritin was determined by the formation of the iron–bathophenanthroline complex [13]. Mitochondria, ~4 mg/ml, were preincubated for 5–7 min at 30°C in a medium of 0.25 M sucrose, 10 mM Hepes buffer (pH 7.40), 2 mM succinate, 0.30 μM ferritin (with ~1200 iron atoms/molecule) and 20 μM bathophenanthroline. The reaction was started by the addition of 40 μM FMN. At the time indicated 0.5 ml aliquots were removed and the iron–bathophenanthroline complex was determined at 530 nm.

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Table 1
Mobilization of iron from ferritin by energy-coupled mitochondria

Addition	Fe(II) (bathophenanthroline)$_3$ (nmol/mg mitochondrial protein)	RC_{ADP} (after incubation)
Ferritin	0.05 (0 –0.09)	2.3 (1.5–2.8)
Ferritin + FMN	0.61 (0.30–0.90)	2.6 (2.3–2.7)

Mitochondria with RC_{ADP} in the range 5–8 were incubated in the presence of ferritin ± FMN as in section 2 except that bathophenanthroline was excluded from the medium. After 10 min anaerobic incubation in the closed chamber of the oxygraph, the RC_{ADP} of the mitochondria was measured by transferring aliquots of the incubation medium to a new medium containing 50 mM glucose, 175 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 5 mM P_i, 2 μM rotenone and 10 mM Hepes buffer (pH 7.40). Further additions were 2.5 mM succinate and 0.6 mM ADP. The amount of iron mobilized was determined by the addition of bathophenanthroline after the incubation was terminated. The results are the means and the ranges (in parentheses) from 4 separate expt.

Protoheme was determined by the pyridine hemochrome method [12,22]. Experimental conditions were as above with 10 μM protoporphyrin IX instead of bathophenanthroline. The spectral shift of safranine induced by the mitochondria was determined in aliquots of the mitochondrial suspension diluted to 1 mg protein/ml in a cuvette containing 32 μM safranine. The change in Δ (A_{524}–A_{554}) was recorded on an Aminco DW 2 UV/VIS spectrophotometer after the addition of 6 μM CCCP [19]. Respiration rates of the mitochondria were recorded by a Clark oxygen electrode (Yellow Springs Instr. Co.) in the medium described (table 1). Glutamate dehydrogenase (EC 1.4.1.3.) was assayed as described [23]. Protein was determined by the Folin-Ciocalteau reagent [24].

3. Results and discussion

As shown in table 1, coupled mitochondria mobilized iron from ferritin. When the mitochondria were incubated with bathophenanthroline or protoporphyrin IX at 2–5 nmol/mg protein, oxidative phosphorylation was maintained although less efficient. Thus, the RC_{ADP} of the mitochondria was reduced ~30% when bathophenanthroline was added (fig.1). The decrease in RC_{ADP} was rapid and caused by a stimulated state 4 respiration while state 3 respiration was inhibited. However, the time-dependent change in RC_{ADP} of mitochondria incubated with bathophenanthroline proceeded at a rate equal to that of the control (fig.1). Protoporphyrin IX behaved essentially as bathophenanthroline while FMN and ferritin had no effect on the RC_{ADP} of the mitochondria. The time progress of the mobilization of iron from ferritin by the mitochondria is shown in fig.2. About 70% of the total amount of iron mobilized was removed from ferritin within the first 10 min of incubation. When protoporphyrin IX was included instead of bathophenanthroline, iron was readily released from ferritin and utilized for the synthesis of protoheme (fig.2). The effect of protoporphyrin IX on the membrane potential of the mitochondria measured as the energy-dependent stacking of safranine [25,26],

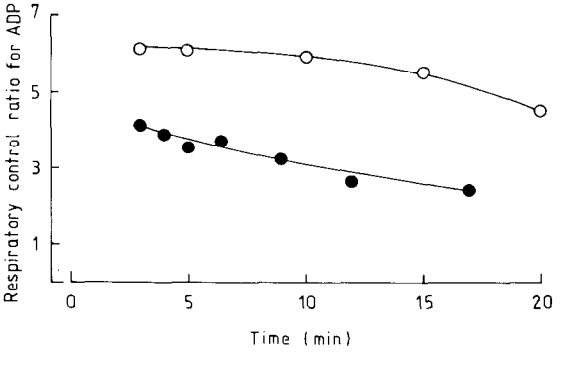

Fig.1. Effect of bathophenanthroline on the RC_{ADP} of the mitochondria. Mitochondria (~1.5 mg protein/ml) were incubated in the oxygraph chamber at 30°C in the absence (○) and in the presence of 8 μM bathophenanthroline (●) in a medium as in table 1. At increasing time intervals 2.5 mM succinate was added to the medium followed 2 min later by 0.6 mM ADP. The time between start of the incubation and the addition of ADP is indicated.

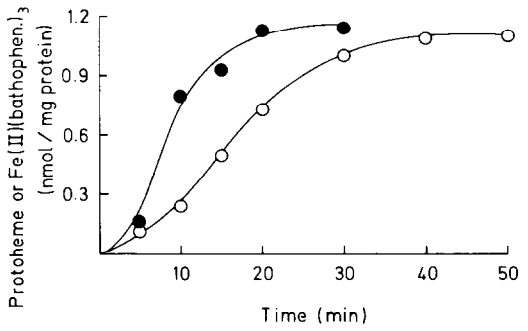

Fig.2. Time course of mobilization of iron from ferritin (•) and of the synthesis of protoheme (○). Experimental conditions as in section 2.

and the change of the energy-state of the mitochondria during synthesis of protoheme with ferritin as iron donor, is shown in fig.3. Analogous to the effect of bathophenanthroline on the RC_{ADP} of the mitochondria, protoporphyrin IX induced a rapid decrease in the membrane potential whereafter the time dependent de-energization of the mitochondria occurred at a rate equal to the control [27]. The ability to generate a membrane potential is a proper measure of the integrity of the mitochondria [28,29], while loss of the membrane potential has been explained by a progressive increase in the permeability of the mitochondrial innermembrane [29]. At a dye/protein ratio equal to that used in the present safranine stacking experiments, the change in absorbance found after 30 min incubation corresponded to a membrane potential of ~60 mV [26]. When at this time samples were withdrawn from the medium and resuspended in aerobic buffer supplemented with succinate for 3–4 min, the membrane potential was increased ~25% as compared to the membrane potential measured immediately after the sample was withdrawn from the medium (fig.3). This indicated that the mitochondria at the time where synthesis of protoheme was completed (fig.2), were sufficiently energized to resist irreversible degradation of the membranes [28]. Moreover, during 30 min incubation with bathophenanthroline or protoporphyrin IX as described in fig.2, <1% of the total glutamate dehydrogenase activity of the mitochondria leaked to the medium; i.e., the structural integrity of the mitochondrial membranes was kept intact. The rate of mobilization of iron from ferritin is a function of the concentration of $FMNH_2$ generated by the mitochondria (table 1) [12–14]. The lower rate of iron mobilization found here compared with [13], is therefore most likely explained by an improved preservation of the mitochondrial membranes under the present experimental conditions as a positive correlation has been found between the rate of reduction of exogenous FMN and the degree of respiration-dependent swelling of the mitochondria [14]. The lower rate of reduction of exogenous FMN in tightly coupled mitochondria with a lower rate of mobilization of iron from ferritin, may partly be compensated by the increased ability of the mitochondria to take up released iron. Thus, it has been shown that uptake of iron by isolated mitochondria is increased with increasing RC_{ADP} values [30]. The sigmoidal time course common to synthesis of protoheme (fig.2), deuteroheme [12] and mobilization of iron from ferritin (fig.2) is basically related to the rate of $FMNH_2$ production; i.e., the initial delay reflects the time needed to reach a concentration of $FMNH_2$ high enough to maintain steady state mobilization of iron from ferritin [14]. The ferrochelatase reaction was delayed compared to the mobilization of iron from ferritin (fig.2). However, 10–12 min after the mobilization of iron was terminated, all iron was trapped by the ferrochelatase and incorporated into protoheme (fig.2). This finding suggests that the ferrochelatase reaction is the rate limiting step in the synthesis of

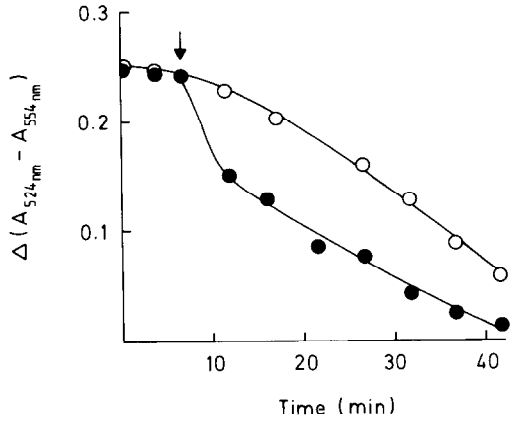

Fig.3. Effect of protoporphyrin IX on the membrane potential of the mitochondria. (•) Mitochondria, ~4 mg protein/ml, were incubated as in section 2. The arrow indicates the addition of 10 μM protoporphyrin IX. (○) Control without protoporphyrin IX. At the times indicated, aliquots were withdrawn from the medium and the energy-dependent stacking of safranine was determined immediately.

heme with ferritin as donor of iron to the mitochondria. The same conclusion was drawn in [21] comparing the rate of synthesis of protoheme using $FeCl_3$ as iron substrate, with the rate of accumulation of protoporphyrin IX by the mitochondria.

In conclusion, this study shows that structurally intact mitochondria with intact oxidative phosphorylation and energy conserving properties are capable of mobilizing iron from ferritin and incorporate the iron into protoheme at a rate which is sufficiently high to satisfy the need for heme in rat liver mitochondria [2,31]. This finding is of importance with respect to the physiological relevance of ferritin as being a possible donor of iron to the mitochondria in situ.

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