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ENERGY-DEPENDENT ACCUMULATION OF IRON BY ISOLATED RABBIT RETICULOCYTE MITOCHONDRIA

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SUMMARY

1. Rabbit reticulocyte mitochondria isolated in a medium of sucrose, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and bovine serum albumin rapidly accumulate iron from the suspending medium when ^{59}Fe(III)–sucrose is used as a soluble and stable model complex.

2. As in liver mitochondria (Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 305, 29–40) the accumulation proceeds by two different mechanisms, i.e. by an energy-independent and an energy-dependent (uncoupler-sensitive) mechanism. The energy-dependent accumulation is inhibited to approx. 80 % by cyanide.

3. The reticulocyte mitochondria possess high- and low-affinity binding sites of iron as recently reported for rat liver mitochondria (Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 325, 38–46). At pH 7.4 the low-affinity sites bind about 110 nmoles of iron per mg of protein with $K'_m \approx 0.3$ mM. The high-affinity sites bind about 28 nmoles of iron per mg of protein with $K'_m \approx 50\,\mu$M, and the binding is completely inhibited by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone.

4. By comparing the energy-dependent accumulation of iron and calcium in mitochondria from reticulocytes and different organs, it is found that iron accumulation (in State 1) relative to calcium (in State 4) is favoured in reticulocytes by a factor of 5 (relative to liver) to 40 (relative to heart).

INTRODUCTION

The mechanisms whereby iron is taken up by immature erythroid cells and incorporated into protoporphyrin IX are only partially understood [1–4]. It is well established, however, that only normoblasts and reticulocytes take up significant amounts of iron [1, 3], and it is generally believed that the transfer of iron from plasma to reticulocytes is mediated by transferrin. Thus, developing normoblasts and reticulocytes contain specific sites at the cell surface for the reversible attachment of transferrin [3], and it has recently been proposed that a transferrin–iron–HCO_3^--

Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCIP, 2,6-dichlorophenol-indophenol; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMS, phenazine methosulfate.

ternary complex is primarily involved in this process [5]. Subsequently HCO_3^- is split off, and from the HCO_3^--free binary complex thus formed, the iron is easily detached by an intracellular iron acceptor [5]. The subsequent intracellular transport processes are so far unknown.

It has been known for some time that as the reticulocyte matures and looses its mitochondria [6], the ability to detach iron from transferrin disappears, and at the same time, the cell becomes unable to take up iron [1, 3]. Evidence for a causal rather than a coincidental relationship between the iron accumulation properties and the presence of mitochondria in the reticulocytes has been presented by Morgan and Baker [3]. They concluded that iron accumulation by reticulocytes was closely linked to energy-yielding processes in the mitochondria. Similar results were reported by Jandl et al. [1] and by Neuwirt et al. [7]. Thus, mitochondria seem to hold a key position in the accumulation and metabolism of iron by erythroid cells. This conclusion is particularly interesting in view of our recent findings of an energy-dependent accumulation of iron by isolated rat liver mitochondria [8].

The present communication deals with results from a study of the accumulation of iron by rabbit reticulocyte mitochondria in vitro. Evidence is presented that the reticulocyte mitochondria possess a very effective mechanism of iron accumulation, possibly related to the specialized function of reticulocytes in heme synthesis.

MATERIALS AND METHODS

Animals and preparation of mitochondria

Adult albino rabbits of both sexes (3.2–3.6 kg of body wt) were fed a standard diet ad libitum and revealed a normal hemoglobin level before reticulocytosis was elicited by repeated bleeding essentially as described by Egyed [5]. The rabbits were fasted overnight and killed by exsanguination on the 5th day using heparin (500 units per kg body wt.) as an anticoagulant (injected intravenously prior to exsanguination). The blood was collected in a pre-cooled beaker (0–2 °C), and immediately centrifuged at a time integral of $(\text{rev./min})^2$ equal to $6.6 \cdot 10^7$ min^{-1} in the swinging bucket HB-4 rotor of Sorvall RC-2B refrigerated centrifuge ($R_{min} = 6.2$ cm, $R_{max} = 14.4$ cm) at 2 °C. The plasma and buffy coat were sucked off, and the red cells washed three times in the standard medium of Schulman [9], using the same centrifugation integral. The final red cell mass was resuspended in 10 times its volume of a medium containing 0.25 M sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer of pH 7.4 and 0.5 % (w/v) bovine serum albumin, and homogenized essentially as described by Guggenheim et al. [10]. The cell sub-fractions were isolated essentially as described for rat liver homogenates [11], except that the medium was supplemented with 0.5 % (w/v) bovine serum albumin. The final mitochondrial pellet was resuspended in 0.25 M sucrose containing 5 mM HEPES buffer, pH 7.4, at a concentration of approx. 10 mg protein per ml. Mitochondria from other tissues (heart, liver, kidney and spleen) were prepared from the anemic rabbits by the standard procedure [11].

Accumulation of iron and calcium

Accumulation of iron, energy-dependent as well as energy-independent, was performed as previously described [8].

Accumulation of calcium was performed essentially as described by Cederbaum and Wainio [12]; mitochondria, approx. 2 mg of protein, were pre-incubated for 10 min at 25 °C in a medium containing in a volume of 1.5 ml: 6.6 mM succinate, 225 mM sucrose, 10 mM HEPES buffer, pH 7.4, 5 mM $MgCl_2$, 5 mM P_i and 10 mM KCl. 0.25 mM $^{45}CaCl_2$ was added, and the reaction was allowed to proceed for 60 s. Aliquots of 1 ml were then withdrawn and transferred to 5 ml ice-cooled incubation medium and centrifuged. The pellet was rinsed with isotonic sucrose, solubilized and taken up in Unisolve and counted in a Mark I Liquid Scintillation Counter (Nuclear Chicago Corp.) [8]. All experiments were run in duplicates.

Respiratory rates

Mitochondrial oxygen consumption was determined with a Clark oxygen electrode (Yellow Springs Instrument's Biological Oxygen Monitor) essentially as described [13].

Enzymic assays and other analytical methods

Acid phosphatase (EC 3.1.3.2) was assayed as described by Richterich et al. [14], and succinate: (PMS)- oxidoreductase (EC 1.3.99.1) as described by Arrigone and Singer [15].

Protein was determined using Folin-Ciocalteau reagent [16].

Determination of hemoglobin, hematocrit and counting of erythrocytes were by standard techniques [17]. Reticulocytes were counted on supravitally stained brilliant cresyl blue smears, and classified according to Heilmeyer [18].

Serum iron and total serum iron-binding capacity were determined by the use of sulfonated bathophenanthroline [19].

Chemicals

ADP, carbonyl cyanide *m*-chlorphenylhydrazone (CCCP), 2,6-dichlorophenolindophenol (DCIP) and phenazine methosulfate (PMS) were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.), $LaCl_3$ from Koch-Light Laboratories Ltd (Colnbrook, England), HEPES (A grade) from Calbiochem (Luzern, Switzerland) and bovine serum albumin, fraction V powder, fatty-acid free from Pentex Biochemicals (Kankakee, Illinois). $^{59}FeCl_3$ and $^{45}CaCl_2$ were purchased from Institutt for Atomenergi, Kjeller, Norway.

Other chemicals were of the highest purity commercially available.

RESULTS

The development of anemia and reticulocytosis during the experimental period is shown in Table I. On the average, by the 5th day, the reticulocytosis amounted to 18.7 % or $37 \cdot 10^4$ reticulocytes per μl blood, and approx. 55 % were classified as belonging to stage O–II. By prolonged bleeding beyond the 5th day, a decrease in the ratio as well as in the number of reticulocytes was observed. Furthermore, there were no significant changes in the concentration of serum iron throughout the experimental period.

The reticulocyte mitochondria contained approx. 7 % of stromal contamination as estimated from the activity of acid phosphatase, and the yields of mitochon-

TABLE I

THE EFFECT OF REPEATED BLEEDING ON THE DEVELOPMENT OF ANEMIA AND RETICULOCYTOSIS IN RABBITS

The rabbits were bled from a marginal ear vein. The numbers refer to the mean and range from experiments with 7 animals. The rabbits were killed by exsanguination at 5 days.

	Time (days)				
	1	2	3	4	5
Blood withdrawn (ml)	38 (32–40)	33 (29–36)	32 (25–36)	28 (25–33)	105 (85–125)
Hemoglobin (g/100 ml)	12.2 (10.4–13.5)	8.8 (6.2–10.6)	6.7 (5.1–8.2)	5.6 (4.2–6.9)	5.4 (4.2–7.8)
Erythrocytes ($\cdot 10^{-6}$ per μl)	5.2 (4.5–6.1)	4.2 (2.9–4.8)	3.4 (2.9–4.6)	2.6 (1.9–3.1)	2.0 (1.8–2.2)
Reticulocytes (%)	1.6 (1.0–2.2)	3.2 (1.5–6.2)	5.2 (3.9–6.8)	9.2 (6.0–14.1)	18.7 (14.1–23.2)
Serum ion (μg/100 ml)	272 (214–303)	245 (158–324)	280 (173–374)	240 (160–361)	286 (181–342)
Total iron binding capacity (μg/100 ml)	468 (328–594)	379 (345–456)	451 (374–557)	425 (397–553)	449 (385–581)

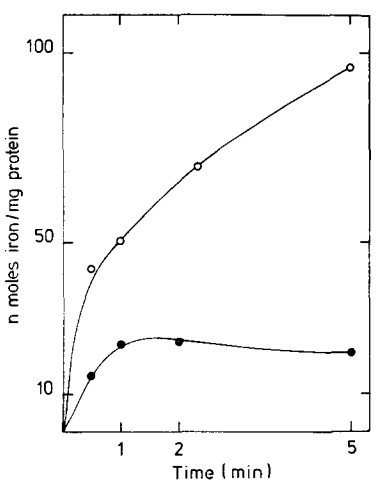

Fig. 1. Time course of iron accumulation in isolated rabbit reticulocyte mitochondria. The mitochondria were suspended in the standard incubation medium (see Materials and Methods) at a protein concentration of 1.8–2.2 mg per ml. The iron concentration was 0.25 mM. ○, iron accumulation in the presence of 17 μM CCCP; ●, the difference between the iron accumulation in the absence and presence of 17 μM CCCP. All points represent the mean of three different experiments.

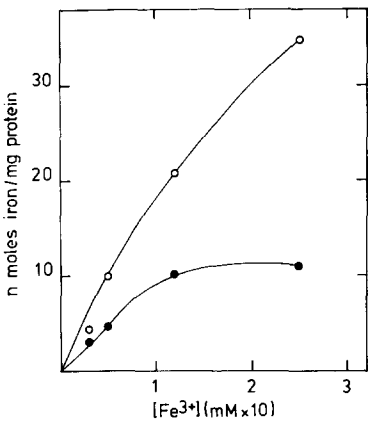

Fig. 2. Effect of iron (III)–sucrose concentration on the iron accumulation in rabbit reticulocyte mitochondria. For experimental details and symbols, see Fig. 1.

dria were only 24–31 %, determined by measuring the activity of succinate : (PMS)-oxidoreductase in the total homogenate and in the isolated mitochondrial populations.

The reticulocyte mitochondria thus isolated were loosely coupled, with respiratory control values (the respiratory rate in the presence of ADP to the rate obtained before the addition of ADP) ranging from 1.4–1.8 with succinate as the substrate. Thus, these mitochondria are expected to operate at a sub-maximal level for other energy-dependent processes as well, including the accumulation of iron. However, in spite of the low respiratory control value with ADP, the accumulation of iron in reticulocyte mitochondria is qualitatively very similar to that recently reported for rat liver mitochondria [8, 11]. Thus, as shown in Figs 1–3 the rabbit reticulocyte mitochondria possess two mechanisms of iron accumulation, i.e. one uncoupler-sensitive (i.e. energy-dependent) and the other uncoupler-insensitive (i.e.

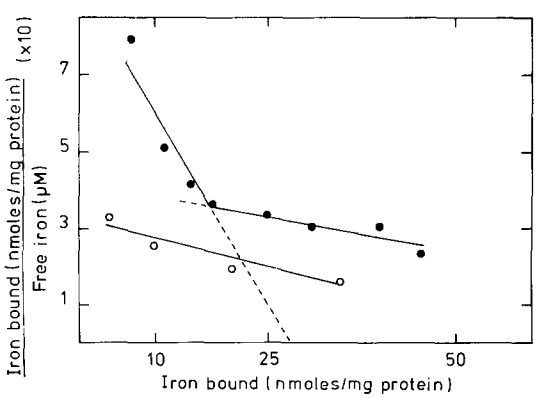

Fig. 3. Effect of iron (III)–sucrose on the iron accumulation in rabbit reticulocyte mitochondria (Scatchard plots). For experimental details, see Fig. 1. ●, total iron accumulation; ○, iron accumulation in the presence of 17 μM CCCP. The mitochondria were preincubated for 10 min with the uncoupler before iron (III)–sucrose was added.

energy-independent). From Fig. 1 it is seen that the uncoupler-sensitive accumulation is a fairly rapid process (i.e. completed within 1–2 min), reaching a saturation level at 20–25 nmoles iron per mg protein. By prolonging the incubation time, however, the amount accumulated tends to decline. On the other hand, the uncoupler-insensitive (i.e. energy-independent) iron accumulation does not reach a saturation level within the time tested.

The concentration dependence is markedly different for the energy-dependent and the energy-independent iron accumulation (Fig. 2). Thus, the energy-dependent accumulation reached a saturation level at approx. 120–150 μM of iron, whereas the energy-independent accumulation did not reach a saturation level in the concentration range tested.

The biphasic nature of the iron accumulation process is evident by plotting the concentration-progress curve in a Scatchard plot (Fig. 3), where the horizontal rectilinear leg corresponds to low-affinity, and the vertical leg to high-affinity binding sites. By extrapolation the number of binding sites was calculated to be 28 and 110 nmoles per mg of protein for the high- and low-affinity binding sites, respectively. In the presence of CCCP, however, the Scatchard plot was monophasic as recently reported for rat liver mitochondria [11].

The energy-dependent accumulation of iron by rabbit reticulocyte mitochondria is reduced by approx. 80 % when respiration is inhibited by cyanide (Table II). This compares favorably with that reported for rat liver mitochondria [8]. Furthermore, both Ca^{2+} and La^{3+} affected the energy-dependent accumulation of iron by reticulocyte mitochondria (Table II) although to a much smaller degree than reported for rat liver mitochondria [11].

In Table III some comparative data from energy-dependent iron and calcium accumulation experiments in mitochondria isolated from various rabbit organs are collected. On a protein basis, the iron accumulation was highest in reticulocyte mitochondria, whereas the calcium accumulation was highest in heart mitochondria. Furthermore, by comparing the ratio $Fe^{3+}_{accumulated} : Ca^{2+}_{accumulated}$ it is seen that accumulation of iron relative to calcium is favoured by a factor of at least 5, relative to

TABLE II

EFFECT OF CYANIDE, La^{3+} AND Ca^{2+} ON THE ENERGY-DEPENDENT IRON ACCUMULATION IN RABBIT RETICULOCYTE MITOCHONDRIA

The mitochondria, 1.2 mg of protein per ml, were incubated in the standard incubation medium (see Materials and Methods). The mitochondria were pre-incubated for 10 min with cyanide, whereas Ca^{2+} and La^{3+} were added simultaneously with 0.25 mM iron (III)–sucrose.

Addition	Concentration	Energy-dependent iron accumulation (nmoles/mg protein)
None*		27.6
CN^-	3.3 mM	5.0
Ca^{2+}	67 μM	27.7
Ca^{2+}	250 μM	15.3
La^{3+}	6 μM	16.5
La^{3+}	33 μM	11.2

* Endogenous respiration.

TABLE III

ENERGY-DEPENDENT ACCUMULATION OF IRON AND CALCIUM BY MITOCHONDRIA ISOLATED FROM RABBIT RETICULOCYTES AND VARIOUS ORGANS (KIDNEY, LIVER, HEART AND SPLEEN)

Mitochondria (at concentrations 1.2–1.8, 3.1–5.9, 2.8–3.4, 1.6–1.8 and 0.8–1.0 mg of protein per ml of reticulocyte, kidney, liver, heart and spleen mitochondria, respectively) were incubated as described (see Materials and Methods) in a total volume of 1.5 ml. Energy-dependent accumulation was measured as defined. All incubations were run in duplicates. The results presented represent the mean of two different experiments. The concentrations of iron and calcium were 0.25 mM. Respiratory rates were determined by incubating the mitochondria (at half the concentrations listed above) in a total volume of 2.5 ml as described. 4.0 mM succinate (in the presence of 4.0 μM rotenone) was used as the substrate, and at steady-state respiration, 1.0 mM ADP was added. Temperature 25 °C.

Mitochondria	Respiratory control values	Ion accumulated (nmoles/mg protein)		Ratio Fe : Ca
		Iron*	Calcium**	
Reticulocyte	1.6	18.3	20.1	0.910
Kidney	3.9	10.5	105.9	0.099
Liver	2.7	8.0	50.5	0.158
Heart	2.9	3.6	174.9	0.021
Spleen	1.6	2.2	34.6	0.042

* Endogenous respiration.
** State 4 respiration.

any other of the mitochondria tested. Here it should be stressed, however, that the ion accumulation experiments were performed on mitochondria in different metabolic states, those with calcium in State 4 and those with iron in State 1. State 4 was selected to increase the energy source available for calcium accumulation in the loosely coupled reticulocyte mitochondria, and State 1 to avoid complex-binding of iron by carboxylates [8].

DISCUSSION

As seen from Table I, no significant changes were observed either in the concentration of transferrin-bound iron or in the degree of saturation of the transferrin throughout the experimental period, which means that the circulating iron depot at any time was sufficiently large to meet the increased demand of the erythropoietic tissue. Thus, the animals were not suffering from iron deprivation, of importance for the ability of the reticulocytes to utilize exogenous iron for hemoglobin synthesis [20].

Reticulocytosis induced by repeated bleeding is generally less extensive than that induced chemically by phenylhydrazine [3, 9, 10, 20, 21]. However, in view of the observed disturbances in the viability of the erythroid cells when phenylhydrazine is used [22], repeated bleeding was preferred in the present study. Furthermore, not only the number, but also the degree of maturation of the reticulocytes are of importance to their function in iron metabolism. Thus, according to Myhre [23], an inverse relationship exists between the degree of maturation of the reticulocytes and the amount of iron accumulated. In the present study reticulocytes of stage O–II amounted to a maximum of $22 \cdot 10^4$ per μl blood. In this preparation the energy-dependent

accumulation of iron was 24.6 nmoles per mg of protein. Although scanty, these findings support the conclusions of Myhre [23].

The loose coupling of the reticulocyte mitochondria observed in the present study is probably due to the isolation procedure which had to be slightly modified for the iron uptake studies. Contrary to the commonly used procedures [7, 10], we had to avoid using chelating agents (EDTA or EGTA) during isolation and incubation [8]. From a ferrokinetic point of view, however, the reticulocyte mitochondria thus isolated compared favorably with the more tightly coupled mitochondria isolated from other organs (Table III).

The qualitative importance of reticulocytes in heme synthesis and iron metabolism has been the subject of several studies in recent years [1–3, 5, 7, 20, 21, 24–28]. Thus, there is ample evidence favouring an energy-dependent transport of iron from the cell surface transferrin binding sites via the cytosol to the ferrochelatase on the matrix side of the mitochondrial inner membrane [1, 2, 4]. The steps involved in this sequence of reactions are so far unknown. In rat liver, however, Moret and coworkers have described phosphoproteins of phosvitin nature as possible candidates for the cytosolic transport of iron [29, 30]. As to the mitochondrial accumulation of iron, this process was until recently considered a passive, energy-independent binding and translocation [12, 31]. From the present results it is evident, however, that reticulocyte mitochondria accumulate iron in a biphasic process, one energy-dependent (uncoupler-sensitive) and one energy-independent. Furthermore, the reticulocyte mitochondria possess high- and low-affinity binding sites, and finally, the time and concentration dependencies of the energy-dependent accumulation are very similar to those obtained with rat liver mitochondria [8, 11]. Thus, the iron accumulation is qualitatively similar in mitochondria isolated from rabbit reticulocytes and rat livers [8, 11]. There are, however, both quantitative and qualitative differences of importance for the specialized function of reticulocyte mitochondria in the biosynthesis of heme and iron metabolism. Firstly, the inhibitory effects of Ca^{2+} and La^{3+} are less than previously observed in rat liver mitochondria [11]. Secondly, in reticulocyte mitochondria the accumulation of iron relative to calcium is favoured by a factor of 5 relative to any other organ tested (Table III). Furthermore, as the ion-accumulation experiments were performed in different metabolic states (see Materials and Methods and Results) the iron: calcium ratios reported probably represent minimum values. Finally, on a protein basis, reticulocyte mitochondria take up approx. 2–3 times the amount of iron taken up by liver mitochondria (Table III). In view of the recent findings of a parallellism between the degree of coupling and the amount of iron accumulated [32], these should be considered minimum values. Thus, by extrapolation to an equal degree of coupling of the liver and reticulocyte mitochondria (see Fig. 2A of ref. 32), the energy-dependent iron accumulation in reticulocyte mitochondria would amount to 50–60 nmoles per mg of protein, thereby increasing the selectivity of the iron uptake several-fold relative to that of liver mitochondria.

Thus, the results reported indicate specialized functions of mitochondria isolated from different tissues at the level of ion transport; i.e. reticulocyte mitochondria possess a mechanism for accumulation of relatively large amounts of iron which is important for the large heme synthesis in these cells [2, 20, 24], whereas heart muscle mitochondria accumulate large amounts of calcium [33–35], which may contribute to maintain a homeostatic control of calcium concentration in the

cytosolic environment and thereby relaxation and contraction of the myofibrils [33, 36, 37].

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