Biochimica et Biophysica Acta, 589 (1980) 162—175

BBA 47786

MITOCHONDRIAL IRON NOT BOUND IN HEME AND IRON-SULFUR CENTERS

ESTIMATION, COMPARTMENTATION AND REDOX STATE

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(Received May 30th, 1979)

Key words: Iron pool; ESR; Bathophenanthroline sulfonate; (Rat liver mitochondria)

Summary

A method is described for the assay of total mitochondrial non-heme iron and a fraction which does not belong to the iron-sulfur proteins (FeS centers) of the outer and inner membrane. The assay of the latter fraction, which is termed 'non-heme non-FeS iron', is based on the formation of a chelate of Fe(II) with bathophenanthroline sulfonate in osmotically swollen mitochondria under conditions where the FeS centers are quite stable as determined by EPR spectroscopy at 20.4 K, 93 K and 123 K.

The 'non-heme non-FeS iron', which in normal rat liver mitochondria amounts to approx. one third of the total mitochondrial iron (i.e. 1.7 ± 0.3 nmol · mg^{-1} protein), does not represent a homogeneous pool of iron. Based on studies of its reaction with bathophenanthroline sulfonate and the dependency of this reaction on reducing agents in mitochondria and mitoplasts, evidence is presented that this non-heme iron is present in two major pools in which the inner membrane constitutes the barrier. A minor fraction (i.e. 0.4 ± 0.2 nmol · mg^{-1} protein) is localized to the 'outer' compartment and a major fraction (i.e. 1.1 ± 0.1 nmol · mg^{-1} protein) is localized to the 'inner' compartment and is equally distributed between the inner membrane and the matrix.

The experiments described in this study also indicate that approximately

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Abbreviations: 'non-heme non-FeS iron' is tentatively used as a term indicating the total amount of mitochondrial non-heme iron related to transport and storage (see Ref. 13); EPR, electron paramagnetic resonance; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

half of the 'non-heme non-FeS iron' of the 'inner' pool is in the ferrous form in mitochondria as isolated, and this was not increased when oxidizable substrates were added to the mitochondria. Although the biological significance of this iron pool is not yet clear, it is likely that it represents a transit iron pool being the proximate iron donor for heme synthesis catalyzed by the enzyme ferrochelatase.

Introduction

The importance of mitochondria in the cellular homeostasis of iron in higher eukaryotes have recently received considerable attention (for review, see Ref. 1). It appears that one of the basic prerequisites for normal heme synthesis is a regulated supply of iron to mitochondria [2]. Thus, the final step in the biosynthesis of heme, i.e. the insertion of Fe(II) into the porphyrin ring, is catalyzed by ferrochelatase (EC 4.99.1.1), a mitochondrial enzyme [3—7] confined to the matrix side of the inner membrane [8—10].

Within the past few years, there has been considerable progress in understanding the uptake and utilization of iron in mitochondria (for review, see Ref. 1). Thus, mitochondria of higher eukaryotes have a transport system to accumulate iron from the environment (cytosol) which has many properties in common with that of calcium, including all of the criteria expected of carrier-mediated transport [1]. It is energy requiring, and when the iron donor is a Fe(III) complex, reducing equivalents are also required [11]. Thus, iron appears to be transported across the inner membrane only as Fe(II) [11,12], but in contrast to the mitochondrial calcium transport, energy is not required to retain iron once it has been accumulated [11]. However, the magnitude of the mitochondrial iron pool related to transport and storage in vivo as well as its compartmentation, chemical nature and redox state are problems that are yet poorly understood.

We have recently demonstrated that rat liver mitochondria contain a substantial amount of non-heme iron which is not related to the iron-sulfur proteins (centers) in the mitochondrial membranes, based on its reaction with bathophenanthroline sulfonate [13]. The purpose of this study is to present more experimental details on this assay system and to demonstrate that the iron measured by this procedure indeed represents exclusively 'non-heme non-FeS iron'. In addition, estimates are also made of the submitochondrial distribution of this iron as well as its redox state.

Materials and Methods

Isolation of mitochondria and mitoplasts. Rat liver mitochondria were prepared as described by Romslo and Flatmark [14] using a medium containing 0.25 M sucrose and 5 mM Hepes at pH 7.4 except that the 10% (w/v) homogenate was centrifuged at $\int_0^t (\text{rev./min})^2 \cdot dt = 8.8 \cdot 10^7\ \text{min}^{-1}$ for sedimentation of the nuclear fraction, which was washed once.

Mitoplasts were prepared by the digitonin method of Chan et al. [15] at

a detergent concentration of 0.2 mg · mg^{-1} protein. The mitoplasts were sedimented by centrifugation at $\int_0^t$ (rev./min)^2 · $dt = 7.0 \cdot 10^8$ min^{-1} and washed twice. A low-speed centrifugation ($\int_0^t$ (rev./min)^2 · $dt = 6.0 \cdot 10^7$ min^{-1}) was included to remove aggregated particles before the mitoplasts were sedimented at $\int_0^t$ (rev./min)^2 · $dt = 7.0 \cdot 10^8$ min^{-1}.

All the centrifugations were carried out in the Sorvall RC-5 refrigerated centrifuge at 4° C, using the swinging-bucket HB-4 rotor (R_{min} = 6.2 cm and R_{max} = 14.1 cm). The final pellets of mitochondria and mitoplasts were resuspended in the isolation medium at a concentration of 65—75 mg protein · ml^{-1}.

Isolation of the mitochondrial matrix fraction. Mitoplasts were resuspended in 5 mM Pipes at pH 6.5 and sonicated in a 'rosette' cell (6 ml) cooled in ice/water. The sonifier (Branson, model S-75) was operated at a current output of 4 A, and the suspension was sonicated five times for 30 s with 1 min intervals in order to keep the temperature below 10° C. The sonicated mitoplasts were centrifuged at $\int_0^t$ (rev./min)^2 · $dt = 9.6 \cdot 10^{10}$ min^{-1} using the 50 Ti rotor (fixed angle, R_{min} = 3.8 cm and R_{max} = 8.1 cm) of the Spinco L-50 ultracentrifuge operated at 4° C. The matrix fraction (supernatant) contained more than 90% of the total mitochondrial malate dehydrogenase (EC 1.1.1.37) activity.

Measurement of mitochondrial swelling. Mitochondrial swelling was measured either by following the change in the apparent absorbance (A') at 575 nm, or by weighing of the mitochondrial pellets obtained by centrifugation [16].

Electron paramagnetic resonance (EPR) spectroscopy. 0.2 ml aliquots of the incubation mixtures were transferred to EPR tubes, frozen in liquid nitrogen and stored at this temperature. EPR spectra were obtained with a Varian V-4502 spectrometer with 100 kHz field modulation. The g values of the various components were identified from the microwave frequency measured by a frequency counter and converter (Hewlett-Packard 5245 L and 5255 A, respectively), from the position of the free radical and from the nominal scan of the field-dial. The desired low sample temperatures were obtained above 77 K by a stream of cold nitrogen gas controlled by a sensor-heater regulatory system (laboratory build equipment) and below 77 K by a helium-flow system (Oxford Instruments SCL 5002 ESR 9) calibrated by means of a carbon resistor with known temperature behavior.

Enzymic assays. Malate dehydrogenase (EC 1.1.1.37) activity was measured spectrophotometrically at 25° C [17]. The incubation medium contained 0.2 mM oxaloacetate, 0.05 mM NADH and 0.1% (w/v) of Triton X-100. Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured spectrophotometrically by following the aerobic oxidation of ferrocytochrome *c* [18] at 25° C. Acid phosphatase (EC 3.1.3.2) activity was measured spectrophotometrically [19] at 37° C. Urate oxidase (EC 1.7.3.3) activity was measured spectrophotometrically [18,20] at 25° C.

Assay of total mitochondrial iron. The mitochondrial content of iron was determined following wet ashing as described by Van de Bogart and Beinert [21].

Assay of heme iron (cytochromes). The cytochromes of the respiratory chain were determined from the difference spectrum (reduced minus oxidized)

of the mitochondrial samples using a dual-wavelength spectrophotometer, essentially as described by Vanneste [22].

Assay of mitochondrial non-heme iron. The total mitochondrial non-heme iron was measured by the bathophenanthroline method [13]. The mitochondria were suspended in a medium containing 10 mM Mes at pH 4.5, 1% (w/v) sodium dodecyl sulfate and excess dithionite (5 μl of a saturated solution) in a total volume of 1 ml. 50 μM bathophenanthroline sulfonate was then added, and the Fe(II)-chelate formation was followed in an Aminco-Chance dual-wavelength spectrophotometer using the wavelength pair 540 nm-575 nm (see Results). The presence of sodium dodecyl sulfate and acid pH (4.5) favors the dissolution of the mitochondrial FeS centers [23—25], and at this pH the color yield of the Fe(II)-bathophenanthroline chelate is still maximal [26].

The mitochondrial 'non-heme non-FeS iron' was also measured by the bathophenanthroline method, but in this case the mitochondria were suspended in a medium containing only 5 mM Pipes at pH 6.5 and excess dithionite (5 μl of a saturated solution) in a total volume of 1 ml. Following a preincubation period of 30 min at 37°C (to complete the hypoosmotic swelling, see Results), 50 μM bathophenanthroline sulfonate was added and the Fe(II)-chelate formation was followed spectrophotometrically (see above).

The amounts of Fe(II) in the unknown samples were calculated from a standard curve obtained by adding known amounts of an iron standard to 1 ml 5 mM Pipes, pH 6.5, in the presence of excess dithionite. Corrections were made for the contribution of iron present in the reagents used which, before use, were all passed over a column of Chelex 100. For the purification of sucrose, a 30% (w/v) solution was adjusted to pH 4 before it was applied to the column. At this pH the stability of the Fe(III)-sucrose complex is low [27] and is also within the pH range where the resin functions satisfactorily [28]. For the purification of dithionite a saturated solution of its sodium salt was passed over the column. The reducing capacity of the solution was unchanged by this treatment as determined by iodimetry [29].

Assay of protein. Protein was determined according to Lowry et al. [30] using bovine serum albumin as a standard.

Animals. Male albino rats (Wistar, MØll) of 200—300 g body weight were fed a standard diet and tap water ad libitum and starved 15—20 h before killing.

Chemicals. Bathophenanthroline and bathophenanthroline sulfonate were purchased from E. Merck (Darmstadt, F.R.G.); Chelex 100 from BioRad Laboratories (Richmond, CA, U.S.A.). A standard solution of iron containing 10.00 μg iron · ml^{-1} was obtained from the G. Frederick Smith Chemical Co. (Columbus, OH, U.S.A.). Other chemicals were of the highest purity commercially available.

Cleaning of glassware. Contaminating iron was removed from the glassware as described by Van de Bogart and Beinert [21].

Results

The contribution of mitochondrial iron to the total iron content of the isolated mitochondrial fraction

Mitochondria, isolated by differential centrifugation in an isoosmotic

medium, are known to be contaminated with other cell organelles, notably lysosomes and peroxisomes [18,31]. A study of the contribution of these organelles to the total amount of iron in the mitochondrial preparation was therefore undertaken.

When the mitochondrial fraction, obtained by the first sedimentation at $\int_0^t (\text{rev./min})^2 \cdot dt = 7.0 \cdot 10^8\ \text{min}^{-1}$, was washed repeatedly, the population of sedimented particles was enriched in mitochondria, having the highest $\overline{s}$ value [18], and the content of lysosomes and peroxisomes approached asymptotically a lower level. From Fig. 1 it is seen that, whereas the specific activity of the marker enzymes acid phosphatase (lysosomes) and urate oxidase (peroxisomes) decreased with increasing number of washings, the total iron content and the specific activity of the marker enzyme cytochrome *c* oxidase (mitochondria) did not change significantly. It can, therefore, be concluded that practically all the iron measured in this preparation is confined to the mitochondria.

Mitochondrial swelling induced by bathophenanthroline and bathophenanthroline sulfonate

From Fig. 2 it is seen that bathophenanthroline induced a rapid change ($t_{1/2} \approx 50$ s) in light scattering when added to mitochondria suspended in an isoosmotic sucrose medium. The swelling, which was of the large-amplitude type, was completed within 25 min at 37° C. Bathophenanthroline sulfonate also induced swelling, but at a markedly slower rate (Fig. 2). That the observed changes in light scattering was due to swelling, was confirmed by weighing of the mitochondrial pellet. The time course of the increase in the mitochondrial pellet weight was comparable with the change in light scattering.

Thus, the reaction between these chelators and 'non-heme non-FeS iron' in

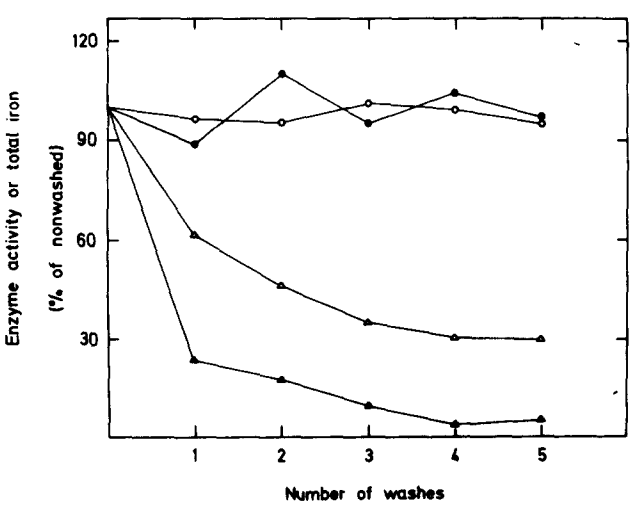

Fig. 1. The effect of repeated washings of the mitochondrial fraction on the total iron content (●), cytochrome *c* oxidase activity (○), acid phosphatase activity (△) and urate oxidase activity (▲). The iron content and enzymic activities were calculated as specific content or activity and expressed in percent of that estimated for the non-washed mitochondrial fraction.

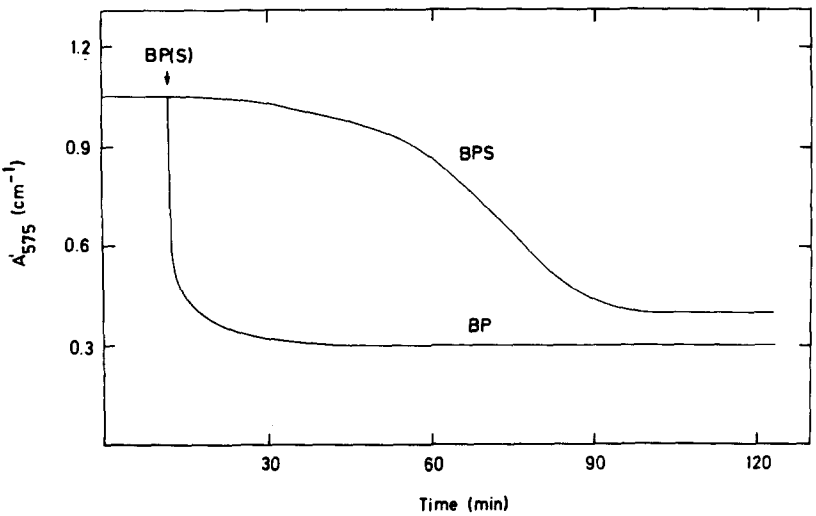

Fig. 2. Changes in light scattering at 575 nm (A'_{575}) of mitochondria induced by 50 μM bathophenanthroline (BP) and 50 μM bathophenanthroline sulfonate (BPS). The mitochondria were suspended in an iron-free 0.25 M sucrose medium containing 5 mM Pipes, pH 6.5, at a protein concentration of 1.0 mg · ml^{-1} in the presence of 5 μM CCCP. The total volume was 1 ml and the temperature 37°C.

mitochondria cannot be studied by dual-wavelength spectrophotometry in an isoosmotic medium. This problem was, however, avoided by preincubation of the mitochondria in a hypoosmotic medium; in this case no additional swelling was induced by the chelators.

Identification of the metal ion chelated on incubation of mitochondria with bathophenanthroline

Bathophenanthroline and its sulfonate derivative are known to form a colored complex with Cu(I) and Fe(II) [32], and in our studies the Fe(II) and Cu(I) complexes were found to have well-separated light absorption spectra (λ_{max} at approx. 535 nm and 425 nm, respectively), and the wavelength pair 540 nm-575 nm was selected for the assay of the Fe(II)-chelate with no interference from *c*-type cytochromes [33,34], *b*-type cytochromes [35] and any Cu(I)-bathophenanthroline sulfonate complex formation.

By treating intact mitochondria with bathophenanthroline and extracting with a suitable organic solvent, chelated metal ions were recovered in the organic phase [21]. The red-colored extract revealed a spectrum which was identical to that of the Fe(II)-bathophenanthroline chelate indicating that the contribution from any Cu(I)-bathophenanthroline complex is negligible, if at all present.

Non-heme iron in rat liver mitochondria

When mitochondria are exposed to sodium dodecyl sulfate and acid pH, their FeS centers are destroyed [23—25], and in addition any 'non-heme non-FeS iron' becomes readily available for reaction with chelating agents. Thus, all the non-heme iron will then react with bathophenanthroline sulfonate following reduction of Fe(III) by dithionite. By this approach the mitochon-

dria were found (Table I) to contain a total amount of 3.1 ± 0.6 nmol · mg^{-1} protein of non-heme iron (mean of four different preparations). This compares well with the figure arrived at by simple calculation from the estimated total mitochondrial iron and heme iron, i.e. 3.0 ± 0.5 nmol · mg^{-1} protein (Table I).

Estimation of mitochondrial 'non-heme non-FeS iron'

The various FeS centers are characterized by their EPR spectra [36], and from such spectra small changes in the iron environment of the FeS centers can be detected [37]. Therefore, the EPR spectra of osmotically swollen mitochondria, incubated for 2 h in the presence and absence of the chelating agent, were compared (Figs. 3—5) at three selected temperatures (i.e. 20.4 K, 93 K and 123 K) since the signal intensity of the various paramagnetic species varies differently with the temperature [36]. It is seen that the EPR spectra with and without chelating agent are practically identical, even for the resonance at $g = 1.89$ (Fig. 5), which originates from the FeS center located to the mitochondrial outer membrane [38], no difference in the signal amplitude was observed in the bathophenanthroline-treated mitochondria. The strong absorption at $g = 1.92$—1.94 has contributions from a number of FeS centers belonging to different dehydrogenases [36], and in all spectra (Figs. 3—5) the change in the peak to peak distance of this line was less than 2% lower in the mitochondria treated with chelating agent as compared to the controls. Based on the EPR spectra (Figs. 3—5) it can be concluded that during the incubation of mitochondria with bathophenanthroline sulfonate no change in the FeS centers occurs, and the iron chelated under these conditions does not originate from the FeS centers.

When bathophenanthroline sulfonate was added to mitochondria suspended in a buffer at pH = 6.5 in the presence of dithionite, a biphasic progress curve was obtained (Fig. 6). The initial rapid phase, which was completed within the mixing time (i.e. approx. 5 s), was followed by a slow, hyperbolic phase which was completed within 3 h. The total amount of iron chelated at that time was 1.7 ± 0.3 nmol · mg^{-1} protein (mean of four different preparations, Table I). As shown above, the FeS iron does not contribute to this reaction, and this iron pool is, therefore, collectively termed 'non-heme non-FeS iron' [13].

TABLE I

THE IRON CONTENT AND MOST COMMON FORMS OF IRON IN ISOLATED RAT LIVER MITOCHONDRIA

Data are in nmol · mg^{-1} protein, mean ± S.D., $n = 4$.

Total	Heme	Non-heme	'Non-heme non-FeS'
4.3 ± 0.6 [a]	1.24 ± 0.08 [b]	3.0 ± 0.5 [c] 3.1 ± 0.6 [d]	1.7 ± 0.3 [e]

[a] Chemical analysis [21].
[b] Determined spectrophotometrically [22].
[c] Determined as the difference between total iron and heme iron.
[d] Determined as the total amount of iron which reacts with bathophenanthroline sulfonate in the presence of SDS and dithionite.
[e] For details, see text.

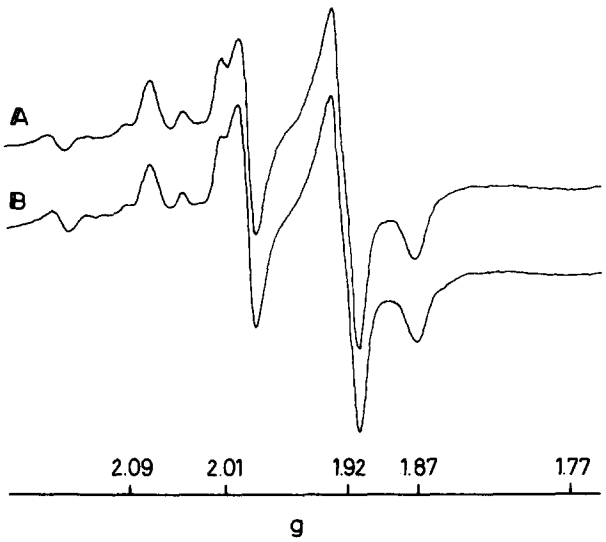

Fig. 3. EPR spectra of intact mitochondria incubated in the presence (A) and the absence (B) of bathophenanthroline sulfonate. Mitochondria were suspended in a medium containing 5 mM Pipes, pH 6.5, at a protein concentration of 133 mg · ml^{-1} in the presence of 5 mM ascorbate, 0.1 mM tetramethyl-*p*-phenylenediamine, 3.3 mM cyanide and 10 μM CCCP. The concentration of bathophenanthroline sulfonate was 1 mM (A). Following incubation for 2 h at 37°C the sample was transferred to the EPR tube. The EPR spectrum was measured using the following conditions: microwave power, 5 mW; modulation amplitude, 12 G; modulation frequency, 100 kHz; scanning rate, 400 G · min^{-1}; time constant, 0.3 s; microwave frequency, 9.2 GHz, and temperature, 20.4 K.

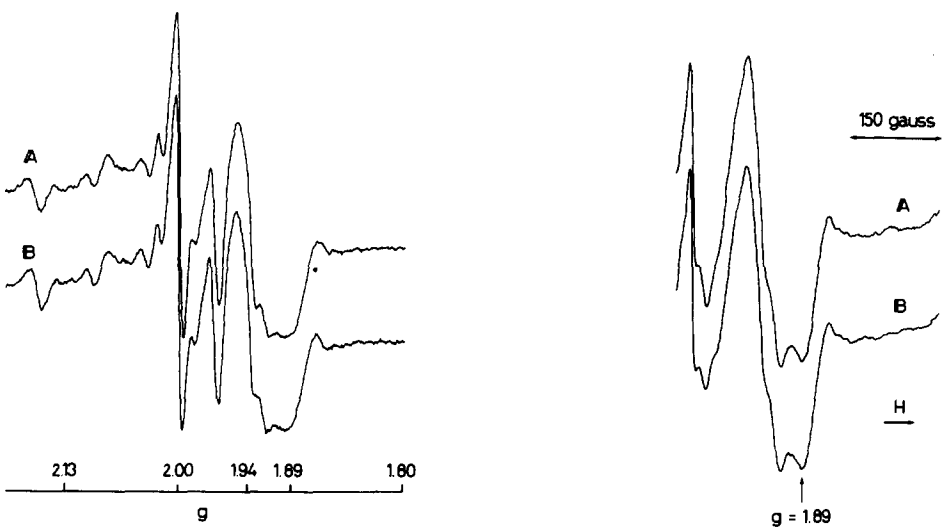

Fig. 4. EPR spectra of intact mitochondria incubated in the presence (A) and absence (B) of bathophenanthroline sulfonate. The samples are the same as those described in the legend to Fig. 3. Conditions of EPR spectroscopy as in Fig. 3 except: microwave power, 25 mW and temperature, 93 K.

Fig. 5. EPR spectra of intact mitochondria incubated in the presence (A) and absence (B) of bathophenanthroline sulfonate. The samples are the same as those described in the legend to Fig. 3. Conditions of EPR spectroscopy as in Fig. 3 except: microwave power, 40 mW, modulation amplitude, 9.6 G, scanning rate, 200 G · min^{-1}; time constant, 1 s, and temperature, 123 K.

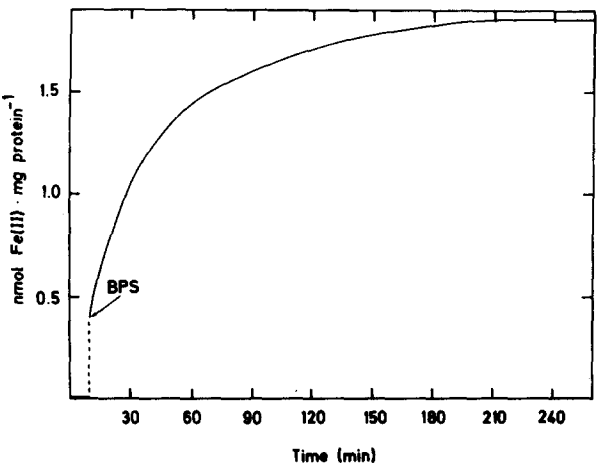

Fig. 6. The time course of the formation of the Fe(II)-bathophenanthroline sulfonate chelate in mitochondria in the presence of a strong reducing agent (dithionite). Mitochondria (1.1 mg of protein) were incubated in 5 mM Pipes, pH 6.5, in the presence of 5 μM CCCP and 5 μl saturated solution and dithionite at 37°C. The total volume was 1.0 ml. At the time indicated bathophenanthroline sulfonate (BPS) was added, final concentration 50 μM. The color development was followed in a dual-wavelength spectrophotometer using the wavelength pair 540 nm-575 nm. The ΔT values were converted to iron concentration from a standard calibration curve obtained by adding known amounts of Fe(II). For experimental details, see Materials and Methods.

Identification of an 'outer' and 'inner' pool of 'non-heme non-FeS iron' in mitochondria

The biphasic progress curve for the formation of the Fe(II)-chelate in mitochondria (Fig. 6), indicates that there may be at least two pools of 'non-heme non-FeS iron' in these organelles. The iron which reacts in the rapid phase, probably represents rather loosely bound iron which is readily chelated under strong reducing conditions (dithionite), whereas the iron chelated in the slow phase probably represents iron more firmly bound to the mitochondria.

To reveal the submitochondrial localization of the 'non-heme non-FeS iron', the chelate formation was studied in mitoplasts under the same conditions as for mitochondria (see legend to Fig. 6). From Fig. 7 it is seen that under strong reducing conditions (dithionite), the amplitude of the rapid phase was significantly reduced, whereas that of the slow phase was only slightly changed from that found in intact mitochondria. This result shows that there are two pools of 'non-heme non-FeS iron' in mitochondria, i.e. one located to the inner compartment (inner membrane + matrix), and the other located to the outer compartment (outer membrane + intermembrane space). These pools of iron will be termed the 'inner' and 'outer' pool, respectively. The 'inner' pool of 'non-heme non-FeS iron' is represented by the total amount of Fe(II) chelated with bathophenanthroline sulfonate in mitoplasts under strong reducing conditions (dithionite), and was found to represent 1.1 ± 0.1 nmol $\cdot$ mg^{-1} protein in mitoplasts (Table II). In mitochondria, the 'outer' pool was estimated to 0.4 ± 0.2 nmol $\cdot$ mg^{-1} protein (Table II).

Whereas the iron of the 'outer' pool of mitochondrial 'non-heme non-FeS

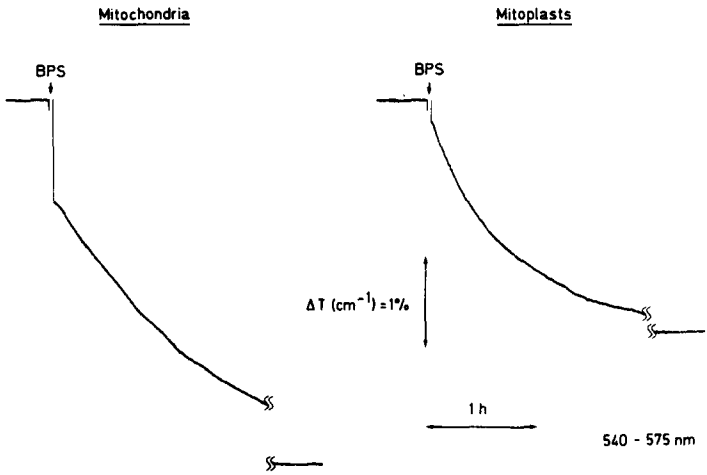

Fig. 7. The Fe(II)-bathophenanthroline sulfonate chelate formation in mitochondria and mitoplasts in the presence of strong reducing agent (dithionite). The conditions were the same as those described in the legend to Fig. 6. The protein concentration was 1.0 mg · ml^{-1} in both experiments.

iron' formed a colored chelate with bathophenanthroline sulfonate only under strong reducing conditions (dithionite), approximately half of the iron of the 'inner' pool did so even in the absence of added reducing agents (Table III). This indicates that whereas the iron of the 'outer' pool is almost totally Fe(III), a significant part (approx. 55%) of the iron in the 'inner' pool is Fe(II) in the mitochondria as isolated. The chelate formation of the iron in the 'inner' pool was not due to photoreduction of the Fe(III)-chelate [39] since the amount of chelate formed was the same in the presence of light and in the dark. When oxidizable substrates were added to the mitoplasts, the fraction of 'non-heme non-FeS iron' of the 'inner' pool chelated by bathophenanthroline sulfonate was slightly lowered (Table III) and amounted to approx. 40% of the total pool.

The Fe(II)-bathophenanthroline sulfonate chelate formation from the mitochondrial 'inner' pool of 'non-heme non-FeS iron' was dependent on pH in the medium. When this pH was decreased from 7.0 to 6.5, the maximal amount of iron, which could be mobilized in the presence of the chelating agent, was increased from approx. 0.5 nmol · mg^{-1} protein to approx. 1.0 nmol · mg^{-1}

TABLE II

THE AMOUNT OF 'OUTER' AND 'INNER' POOL OF 'NON-HEME NON-FeS IRON' IN MITOCHONDRIA AND MITOPLASTS

Data are in nmol · mg^{-1} protein, mean ± S.D., n = 3. For methods of estimation, see text.

	Total	'Outer' pool	'Inner' pool
Mitochondria	1.8 ± 0.5	0.4 ± 0.2	1.4 ± 0.4 [a]
Mitoplasts	1.1 ± 0.1	— [b]	1.1 ± 0.1

[a] Estimated from the slow phase of the Fe(II)-bathophenanthroline sulfonate formation in mitochondria in the presence of dithionite, cf. Fig. 6.

[b] The 'outer' pool is negligible in the mitoplast preparation, see text.

TABLE III

THE AMOUNT OF Fe(II)-BATHOPHENANTHROLINE SULFONATE FORMED IN MITOPLASTS UNDER DIFFERENT REDUCING CONDITIONS

The Fe(II) of the 'non-heme non-FeS iron' was measured using the bathophenanthroline sulfonate method as described in the legend to Fig. 6. Mitoplasts (approx. 1 mg of protein) were suspended in 1 ml buffer (5 mM Pipes, pH 6.5) in the presence of 10 μM CCCP. The concentrations of succinate, malate and glutamate were all 5 mM. 5 μl of a saturated solution of dithionite was used. Data are in nmol · mg^{-1} protein, mean ± S.D., n = 6.

Reducing condition	Fe(II)-bathophenanthroline sulfonate formed
Endogenous substrates only	0.51 ± 0.11
Succinate, malate and glutamate added	0.36 ± 0.14
Dithionite added	0.93 ± 0.12

protein (Fig. 8). Decreasing the pH further to 6.0 did not increase the Fe(II)-chelate formation (Fig. 8).

Discussion

Previous studies on the total iron content and the non-heme iron compounds of rat liver mitochondria have shown that these organelles contain a significant amount of non-heme iron which is not related to the FeS proteins in the different mitochondrial compartments [13]. The assay of this non-heme iron pool was based on its reaction with bathophenanthroline sulfonate in osmotically swollen mitochondria. In the experiments reported in this paper, we have presented further evidence for the specificity of the reaction on which the assay of this iron pool is based.

The present studies show that the reaction of bathophenanthroline sulfonate with Cu(I) does not contribute to the absorbance changes measured using

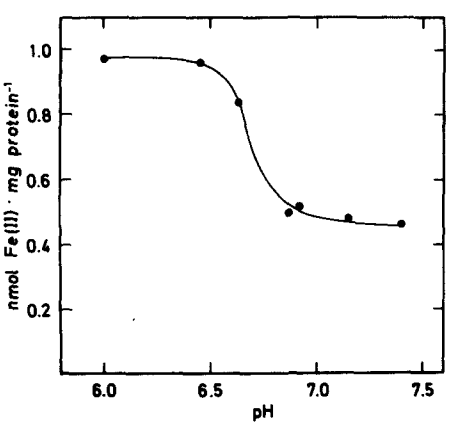

Fig. 8. Effect of pH in the medium on the degree of mobilization of iron from the mitochondrial 'inner' pool of 'non-heme non-FeS iron'. The extent of Fe(II)-bathophenanthroline sulfonate chelate formation in the slow phase (cf. Fig. 6) was measured at the end of the reaction (t = 3 h) under the conditions stated in the legend to Fig. 6, except that the pH of the mediun was varied and measured at the end of the incubation period.

absorbance difference ΔA(540 nm-575 nm). The standard assay procedure is therefore selective for Fe(II). Furthermore, it is well documented that heme iron is not chelated by bathophenanthroline sulfonate [40,41]. The key question, therefore, is the possible implication of the FeS proteins (centers) in the reaction.

Several lines of evidence support the conclusion that the iron of the various FeS centers present in the mitochondria is not chelated by bathophenanthroline sulfonate in the procedure outlined here. First, it has been shown earlier that isolated and membrane-bound FeS proteins are rather stable towards reaction with chelating agents at neutral pH and in the absence of protein-denaturing agent [42,43]. Secondly, the pH dependence of the reaction of mitochondrial iron with the chelating agent (Fig. 8) differs from the effect of pH on the stability of FeS proteins [25]. Thus, whereas the mobilization from the former was maximal when the pH was decreased to approx. 6.5 (Fig. 8), the FeS centers of both ferredoxins and high-potential iron-sulfur proteins are stable until the pH is lowered to pH 6.0 and the dissolution increases progressively below this value [25]. Furthermore, the FeS centers of the mitochondrial inner membrane are not affected when incubated at pH 6.0 for 1 h at 23°C [44], and from Fig. 5 it is seen that following incubation of the mitochondria at the conditions used in the present study (i.e. 37°C for 2 h, pH 6.5) an EPR signal at $g = 1.89$, representing the outer membrane FeS center is clearly observed, and the signal is not influenced by the presence of bathophenanthroline sulfonate during the incubation (Fig. 5). The most convincing evidence, however, is that none of the EPR spectra of the mitochondria exposed to the chelating agent for 2 h at 37°C recorded at three selected temperatures differ from the spectra of the controls (Figs. 3—5). Since these spectra are able to detect even minor changes in the environment of the FeS centers [37], it is concluded that these centra are not affected by bathophenanthroline sulfonate at our standard incubation conditions.

The conclusions to be drawn from these findings are that the Fe(II)-bathophenanthroline sulfonate chelate is formed primarily by mobilization of a mitochondrial non-heme iron pool which does not include the FeS centers of the outer and inner membrane. The present studies also show that there is a compartmentation of this mitochondrial 'non-heme non-FeS iron'. Of particular interest is the finding that the major fraction of this iron is confined to the mitochondrial inner compartment, i.e. matrix and inner membrane. Although the biological significance of this iron pool is not yet known, it is likely that it represents a transit iron pool being the proximate iron donor for the conversion of porphyrin to heme catalyzed by the enzyme ferrochelatase [13]. Numerous studies have indicated that this enzyme is exclusively located in the mitochondria [3—7], confined to the matrix side of the inner membrane [8—10], and a continuous supply of Fe(II) is required for heme synthesis. The experiments described in this study indicate that more than 50% of the 'non-heme non-FeS iron' of the 'inner' pool is in the ferrous form in mitochondria as isolated, and the finding that exogenous substrates (carboxylates) slightly lowered the fraction of Fe(II) chelated by bathophenanthroline sulfonate (Table III) may be due to binding of iron as carboxylate complexes [45] interferring with the formation of the Fe(II)-bathophenanthroline sulfonate chelate. The exact

chemical nature of the mitochondrial iron-binding ligands is not known. However, regardless of their chemical nature, the fact that the major fraction of the iron appears to exist in the ferrous form suggests that it is bound in a rather specific manner giving the iron a sufficiently high redox potential to stabilize the reduced form. This is important from a physiological point of view since only Fe(II) can be utilized in heme synthesis (for review, see Ref. 1).

Acknowledgements

This work was supported by Grants-in-Aid from the Norwegian Research Council for Science and the Humanities (C.11.14-3) and the Norwegian Cancer Society.

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