The Utilization of Iron and its Complexes by Mammalian Mitochondria

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Sonicated mitochondria catalyse the reduction of ferric salts, and the subsequent incorporation of Fe^{2+} into haem, when provided with a reducing substrate such as succinate or NADH. The rate of haem synthesis was low under aerobic conditions and, after a short lag period, accelerated once anaerobic conditions were achieved; it was insensitive to antimycin A. The lag period was decreased by preincubating the mitochondria with NADH and Fe^{3+} . Newly formed Fe^{2+} was autoxidized rapidly and the consequent O_2 uptake was measured with an oxygen electrode to determine the rate of enzymic formation of Fe^{2+} from FeCl_3 ; this reaction was rapid in sonicated mitochondria provided with NADH or succinate and was insensitive to antimycin A. The reaction was very slow in intact mitochondria, suggesting a permeability barrier to Fe^{3+} ions. This system was used to test the permeability of the mitochondrial membrane to various iron complexes of biological importance. Of the compounds tested only ferrioxamine G appeared to penetrate readily and the iron of this complex was reduced when intact mitochondria were supplied with succinate or NADH-linked substrates. The reduction was insensitive to rotenone or antimycin A. Both ferrioxamine G and ferrioxamine B were, however, reduced by particles. The membrane fraction of sonicated mitochondria was necessary for the reduction. The rate of ferrioxamine B reduction by sonicated mitochondria was measured by a dual-wavelength spectrophotometric assay and was found to be stimulated in conditions where the Fe^{2+} produced was utilized for haem synthesis. The addition of FeCl_3 to anaerobic particles caused an oxidation of cytochrome *b* when this region of the respiratory chain was isolated by treatment with rotenone and antimycin A. These results suggest that the reduction of ferric iron and its complexes occurs inside the inner mitochondrial membrane in proximity to ferrochelatase. Possible sites for this reduction are the flavoproteins, succinate and NADH dehydrogenase.

Mitochondrial electron-transport systems involve two classes of iron compound, the cytochromes and the non-haem iron proteins. Storage forms of iron, such as ferritin, may also be found in mitochondria. The enzyme ferrochelatase, which participates in the insertion of iron into porphyrins, is located on the inside of the inner mitochondrial membrane (Jones & Jones, 1969) and utilizes iron ions only in the reduced form (Porra & Jones, 1963). Thus the utilization of iron by mitochondria for the synthesis of haem presents two problems: how does iron cross the ion-impermeable inner membrane, and how is Fe^{2+} produced either from ferric iron as the free ion, which is very insoluble in aqueous buffers at pH 7.0 (Schubert, 1964), or from possible storage complexes or other complexes of Fe^{3+} . Ferrous iron is unstable and rapidly autoxidized at physiological pH values under aerobic conditions. The known storage forms of iron, ferritin and haemosiderin, are ferric com-

plexes, as also is transferrin, the protein complex in which iron is transported in the plasma.

It is particularly difficult for cells to assimilate iron because of the insolubility of ferric hydroxide around neutral pH values. Further, there is increasing evidence that in micro-organisms special mechanisms have been developed for solubilizing iron and then transporting the metal through the plasma membrane. It was shown by Neilands (1952) that the smut fungus *Ustilago sphaerogena* produces an iron chelate, ferriochrome, when grown in an iron fermenter; when grown in iron-limited conditions this organism excretes the metal-free chelate desferri-ferriochrome, and it was proposed by Neilands (1957) that ferriochrome was involved in iron transport.

Compounds similar to ferriochrome, in which the active iron-binding site contains hydroxamate groups, have been found in many actinomycetes and fungi (see, e.g., Prelog, 1964; Atkin *et al.*, 1970) and a wide range of iron-chelating materials have been isolated from low-iron cultures of bacteria and mycobacteria (see, e.g., Snow, 1970; Wang & Newton, 1969).

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J. B. Neilands (unpublished work, cited by Atkin *et al.*, 1970) has speculated that most, or all, aerobic micro-organisms require or synthesize low-molecular-weight iron-transport agents. These may interact with specific permeases within the membrane (Wang & Newton, 1971). For mammalian cells there is little evidence as to the mechanism by which iron is transported from the outside of the plasma membrane (which it reaches as the iron-complex form of transferrin) through the cytosol and across the inner mitochondrial membrane.

In the present paper, we describe methods that permit the measurement of the permeability of mitochondrial membranes to ferric iron and some of its complexes and report the specificity of the processes that reduce ferric iron complexes to yield the ferrous ions needed for haem synthesis. Some evidence about the localization of the iron-reducing sites is also discussed.

Experimental

Methods

Preparation of mitochondria. Conventional methods (see Chappell & Hansford, 1969) were used to prepare mitochondria from rat livers and blowfly flight muscle.

Uptake of O_2 . Consumption of O_2 was measured polarographically by using a Clark-type oxygen electrode in a 5.5 ml reaction vessel in a jacket at 36°C.

Absorption spectra. Difference spectra were obtained with a split-beam spectrophotometer (Yang & Legallais, 1951) constructed by using a Hilger D 330 monochromator.

Assay of ferrochelatase. An account of the dual-wavelength spectrophotometric determination has been given elsewhere (Jones & Jones, 1969, 1970) and where the incubation mixture has been modified, details are given in the text. As a routine deuteroporphyrin was used as the porphyrin substrate, as it gave faster rates than protoporphyrin, the presumed natural substrate, and was also more soluble in the buffers used. All determinations were carried out at 37°C.

Special chemicals

Deuteroporphyrin IX, obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., was dissolved in a minimal quantity of 2M- NH_3 and a 1 mM stock solution was prepared in 40 mM-tris, adjusted to pH 8.2 with HCl, and containing 2% (v/v) Tween 80. Ferrioxamine G, ferrioxamine B, ferrioxamine D₁, ferrirubin, ferrichrome, ferrichrome A and ferrichrysin were generous gifts from Professor W. Keller, Technische Hochschule, Zurich, Switzerland. Ferritin was obtained from Calbiochem, London

W.1, U.K. Ferredoxin, prepared from leaves of the vegetable marrow (*Cucurbita pepo*), was a gift from Dr. E. J. Hewitt, Research Station, Long Ashton, Bristol, U.K. Imferron was a gift from Fisons Pharmaceuticals Ltd., Loughborough, Leics., U.K. Transferrin was a gift from Dr. J. Williams of this Department.

Results

Salts of ferrous iron are rapidly autoxidized and would not persist in the aerobic environment where ferrochelatase normally functions. It appeared likely that mitochondria have a mechanism for producing the Fe^{2+} ions for ferrochelatase from ferric iron or its complexes. A progress curve for haem synthesis when $FeCl_3$ is added to a cuvette containing sonicated mitochondria strongly supports this view (Fig. 1). When a respiratory substrate (NADH or succinate) was provided, as well as iron and deuteroporphyrin, the rate of haem synthesis was initially small. After the onset of anaerobiosis, indicated by the sudden rise in the trace caused by the 'tail' of spectroscopic changes accompanying cytochrome reduction (Jones & Jones, 1970), there was a short lag period and then haem synthesis continued at an accelerating rate. No

Fig. 1. Time-course of metalloporphyrin formation and the effects of air and antimycin A on haem formation by sonicated mitochondria

Ferrochelatase activity was determined as described in the Experimental section. Sonicated mitochondria (approx. 8 mg of protein), succinate (20 μ mol), deuteroporphyrin (100 nmol) and Fe^{3+} or Co^{2+} (1.0 μ mol) were incubated in 2.5 ml of 0.1 M-tris-HCl buffer, pH 7.2, containing 0.1% (v/v) Tween 80. The change in extinction $\Delta(E_{498} - E_{511})$ was recorded from the time of mixing. (a) Co-porphyrin formation; (b) haem formation; (c), haem formation with antimycin A (10 μ g) added at arrow; (d) as for (c) but with vigorous oxygenation at the time of addition of antimycin A; (e) $\Delta(E_{498} - E_{511})$ in the absence of added metal. Numbers in parentheses denote $10^2 \times \Delta(E_{498} - E_{511})/\text{min}$.

haem synthesis occurred in the absence of the respiratory substrates succinate or NADH. Although haem formation continued in the presence of respiratory inhibitors such as antimycin A, the introduction of air resulted in an inhibition. Oxygenation of antimycin-containing incubation mixtures, where the rate of removal of O_2 by respiratory activity was slow, gave conditions where the process of autoxidation appeared to decrease the concentration of Fe^{2+} available for ferrochelatase. Under these aerobic conditions a low but significant linear rate of haem synthesis was found. If ferrous iron was added as substrate, maximum rates of haem synthesis were observed only after anaerobic conditions had been achieved within the cuvette after the oxidation of NADH or succinate. There was no requirement for NADH or succinate when Co^{2+} was the metal substrate (Fig. 1).

As shown in Fig. 1 there is a slow 'drift rate' measured on the recorder even in the absence of added iron. This 'drift', which was essentially negligible after 5 min, required the presence of both mitochondria and deuteroporphyrin. We are not certain of the explanation for this 'drift', but have found that rapid changes in extinction were induced by adding solutions of bovine plasma albumin to

Fig. 2. Effect of varying the order of addition of reactants on the rate of haem formation by sonicated mitochondria

Sonicated mitochondria (8mg of protein) were incubated in 2.5ml of 0.1M-tris-HCl buffer, pH7.2, containing 0.1% (v/v) Tween 80, with two of the three substrates, NADH (2.5 μ mol), deuteroporphyrin (100nmol) and $FeCl_3$ (1.0 μ mol). The reaction was started after 5min preincubation by the addition of deuteroporphyrin (a), Fe^{3+} (b) or NADH (c). For curves (d) Fe^{3+} or NADH was omitted. Numbers in parentheses denote $10^2 \times \Delta(E_{498} - E_{511})/\text{min}$.

porphyrin solutions and that the soluble fraction rather than the membrane fraction of mitochondria was involved. It appears that some non-specific binding of porphyrin to soluble protein took place in the first few minutes of incubation, resulting in a decrease in the extinction of the porphyrin. Nevertheless, after 3–5 min the rate of 'drift' did not contribute significantly to the measurement of ferrochelatase activity.

When the sequence of addition of porphyrin, Fe^{3+} and oxidizable substrate was varied, noteworthy differences were found both in the maximum rate of haem formation and in the time taken ('lag') to achieve the maximum rate (Fig. 2). Incubation of the mitochondria with porphyrin and Fe^{3+} before addition of NADH resulted in the lowest activity and the longest lag. Incubation with Fe^{3+} alone had a detrimental effect on subsequent ferrochelatase activity.

Fig. 3. Effect of protein concentration and of preincubation of mitochondria with Fe^{3+} and NADH on the time taken to reach maximum ferrochelatase activity (lag time)

Ferrochelatase activity was determined as stated in the Experimental section. Sonicated mitochondria [4 (Δ) or 10 ($\circ$) mg of protein], NADH (2.5 μ mol) and $FeCl_3$ (1 μ mol) were incubated in 2.5ml of 100mM-tris-HCl buffer, pH7.2, containing 0.1% Tween 80 for various times at 37°C. The reaction was started by the addition of deuteroporphyrin (100nmol) and the $\Delta(E_{498} - E_{511})$ was recorded.

Fig. 4. Mitochondrial reduction of Fe^{3+} measured by autoxidation of the Fe^{2+} produced

The rate of O_2 consumption was measured as described in the Experimental section. The incubation medium contained KCl (80mM) and tris-HCl buffer (20mM), pH7.2. Additions were sonicated mitochondria (8mg of protein) or intact mitochondria (4mg of protein), succinate (4mM), P_i (4mM), ADP (0.2mM), antimycin A (10 μg), Fe^{3+} (1mM) and EDTA (2.5mM). The final volume was 5.5ml and the temperature was 36°C. (a) Sonicated mitochondria; (b) intact mitochondria.

The presence of the oxidizable substrate (but not porphyrin) prevented this damaging effect. This protective action of NADH was manifested only in the presence of mitochondria and was apparently not due to simple effects on iron or porphyrin. Prolonged preincubation of mitochondria with NADH alone had very little effect on the lag time. The shortest lag times and maximum rates were obtained when mitochondria were preincubated with NADH and Fe^{3+} before addition of porphyrin. Results of a more detailed study of the effect of preincubation of mitochondria with both NADH and Fe^{3+} on the lag time are shown in Fig. 3. The relationship between the lag time and the different times of preincubation was linear over the first 16min. Newly formed Fe^{2+} could possibly be non-specifically adsorbed to mito-

chondrial protein and made unavailable to ferro-chelataze until all such binding sites were filled. This would result in a lag in the reaction. However, it was found that the lag period was inversely related to the amount of mitochondrial protein present (Fig. 3). This observation indicates that the lag period is not likely to be due to simple adsorption of substrate Fe^{2+} .

Measurement of Fe^{3+} reduction with an oxygen electrode

Jones (1968) showed that at pH8.2 the autoxidation of FeSO_4 in aqueous buffer systems was a rapid process that could readily be measured with an oxygen-electrode assembly. It seemed possible that this reaction could be used in assaying the reduction of Fe^{3+} by a mitochondrial system, since the Fe^{2+} formed should be rapidly autoxidized and the resulting O_2 consumption could be measured. Fig. 4(a) shows that if the respiration of succinate by mitochondrial particles was inhibited by the addition of antimycin A, O_2 uptake was indeed restored by the addition of FeCl_3 . Once again there was a lag phase observed after the addition of the Fe^{3+} , and the initial O_2 -uptake response tended to be sigmoidal, although a subsequent addition of Fe^{3+} produced a rapid linear rate. This rate of O_2 uptake was diminished by the chelating agent EDTA. We interpret this curve to mean that the normal flux of electrons from succinate to O_2 is blocked by antimycin A, but that Fe^{3+} acts as an alternative electron acceptor; the Fe^{2+} that is formed then reacts rapidly with O_2 giving an apparent resumption of respiration. Although this should result in a re-cycling reaction with constant regeneration of Fe^{3+} , the very low solubility of iron salts at neutral pH values may lead to a loss of available iron from the system. When this reaction was attempted with intact mitochondria (shown by their response to ADP to have good respiratory control) there was only a slight response to the addition of FeCl_3 (Fig. 4b). We interpret this difference in response to Fe^{3+} between intact and sonicated mitochondria as a result of the impermeability of the intact mitochondria to Fe^{3+} ions. It therefore follows that the site of reduction of Fe^{3+} by succinate must be located inside the inner mitochondrial membrane. It is not possible to use this technique to measure the reduction of Fe^{3+} by NADH or NADH-linked substrates in sonicated mitochondria, since in these preparations NADH may be oxidized via cytochrome b_5 of the outer mitochondrial membrane in a sequence of reactions that is insensitive to the commonly used inhibitors rotenone or antimycin A (see Scheme 1). In intact mitochondria respiring glutamate+malate, where this by-pass is not operational, the response to added Fe^{3+} was slow, suggesting that if NADH-linked sub-

Scheme 1. *Electron transport from NADH and succinate in sonicated mitochondria*

Uq indicates ubiquinone, Fp indicates flavoprotein and Cyt. indicates cytochrome. X indicates a postulated intermediate (see the text).

strates could be used to reduce Fe^{3+} , the site of reduction was inside the inner mitochondrial membrane. Evidence in support of this suggestion is given in the studies with penetrant iron complexes described below.

A number of iron complexes were studied to determine whether they were capable of penetrating the mitochondrial membrane to the site of iron reduction. Some gave positive results (Fig. 5; Table 1). Ferrioxamine G restored O_2 uptake that had been inhibited after the addition of antimycin A to mitochondria respiring succinate or glutamate+malate. When glutamate+malate was the substrate, inhibition of O_2 uptake could be caused by adding rotenone. This inhibition site also could be by-passed and O_2 uptake restored by the addition of ferrioxamine G. Ferrioxamine B was slightly effective in restoring O_2 uptake, whereas ferrioxamine D₁ was without effect. The activity of various iron complexes in restoring O_2 uptake to intact and sonicated mitochondria inhibited by treatment with antimycin A and also their activity as a source of iron for haem synthesis are given in Table 1. The only compound with good activity in all three categories is ferrioxamine G. The storage and transport forms of iron in mammals, ferritin and transferrin, were inactive in all three systems.

Further evidence for the ready penetration of ferrioxamine G is shown in Fig. 5(b) where its effect in stimulating the O_2 uptake of coupled mitochondria resembles that of the known penetrant Ca^{2+} . The declining rate of O_2 uptake indicates that the iron of ferrioxamine G is not freely recycled after its reduction and autoxidation. A second addition of ferrioxamine G leads to a similar loop of O_2 uptake. The Fe^{3+}/O ratio in such experiments was about 6.0.

Both the maximum rate of haem synthesis by sonicated mitochondria and the time taken to achieve this maximum rate were influenced by a variety of factors including concentrations of enzyme, iron and respiratory substrate. The dependence on the concentration of iron and iron chelates added to the incubation mixture is shown in Figs. 6(a) and 6(b). In each case FeCl_3 was more effective than equal concentrations of the ferrioxamines. K_m values of approx. $200\text{ }\mu\text{M}$ for FeCl_3 and 1 mM for ferrioxamine G were found (Figs. 6a and 6b). When the maximum rates of iron-induced O_2 uptake and the time taken to attain these maximum rates were determined as a function of the concentration of added iron (Figs. 7a and 7b), the rates of O_2 uptake were found to continue to increase over the range of concentrations used.

When sonicated mitochondria were centrifuged and the particulate fraction was assayed for the capacity to form haem from added Fe^{3+} and porphyrin, with succinate or NADH as respiratory substrates, it was found that the loss of the soluble fraction did not significantly inhibit the process, i.e. none of the enzymes involved in the Fe^{3+} reduction was in the mitochondrial matrix (Table 2). The soluble fraction was required, however, if citrate or isocitrate was the respiratory substrate. This would be expected, since the enzymes involved in the tricarboxylate cycle are located in the soluble matrix fraction.

When the maximum rates of haem synthesis by mitochondrial sonicates with FeCl_3 were determined with succinate or NADH as electron donors it was found that higher rates were given by NADH. Addition of succinate to the NADH-driven reaction did not increase the rate above that given by NADH

alone except when NADH concentrations were sub-optimum (Fig. 8). The K_m for NADH, determined as an average of several experiments, was $100\mu\text{M}$, which is similar to that found for mitochondrial NADH dehydrogenase (Singer & Gutman, 1971).

The oxygen-electrode method for measuring the reduction of iron is indirect, and a more direct assay was required to solve certain problems. By observing the colour change when the ferrioxamines were reduced it was possible to measure their reduction directly. To make these measurements during haem synthesis it was necessary to compensate for the absorption changes taking place when the highly coloured porphyrin was used up and haem was

Fig. 5. Stimulation of O_2 uptake of intact respiring mitochondria by ferrioxamine G in the presence (a) and the absence (b) of respiratory inhibitors

The rates of O_2 consumption were measured as described in the Experimental section. The incubation medium contained KCl (80mM) and tris-HCl buffer (20mM), pH 7.2. Additions were intact mitochondria (6.5mg of protein), glutamate (4mM) + malate (4mM), or succinate (4mM), P_i (4mM), ADP (0.2mM), ferrioxamine G (1mM), antimycin A ($10\mu\text{g}$) or rotenone ($1\mu\text{M}$). The final volume was 5.5ml and the temperature was 36°C .

formed. The spectroscopic changes involved (Fig. 9) are such that it is possible to use a dual-wavelength assay for ferrioxamine reduction by selecting wavelengths that are isosbestic for the changes in absorption of porphyrin and haem. The wavelengths selected were 581.4nm and 480nm. By using these wavelengths it was shown that ferrioxamine B, selected for study because of its relative abundance, was reduced very much faster when porphyrin was present to utilize the reduced Fe^{2+} compound, although the rate of haem synthesis was lower than the rate of iron reduction (Fig. 10). The 'lag' in the reduction of ferrioxamine B, noted in the oxygen-electrode assay, was also found in this spectrophotometric assay. It must be remembered, however, that these assays are not strictly comparable, because the spectrophotometric assay is carried out under anaerobic conditions. Lack of interference in the measurement of ferrioxamine reduction by porphyrin changes was confirmed in separate experiments where FeCl_3 was the iron source; no increase in $\Delta(E_{581.4} - E_{480.4})$ was then found.

The oxygen-electrode assay had shown that ferrioxamine G reduction was insensitive to rotenone when NADH-linked substrates were used and was insensitive to antimycin A when succinate was the substrate, suggesting the possibility that there are at least two possible sites of interaction between reducing systems and Fe^{3+} . It was possible that some separate reduction mechanism was involved, with the special function of supplying Fe^{2+} for haem synthesis, and that this reduction system was not associated with the respiratory chain. A direct interaction with mitochondrial electron transport is, however, suggested by the experiment illustrated in Fig. 11. Sonicated mitochondria were allowed to become anaerobic with NADH as substrate, in the two cuvettes of a split-beam spectrophotometer. Antimycin A and rotenone were added to both cuvettes in an attempt to isolate the 'classical' cytochrome *b* region of the respiratory pathway, and then FeCl_3 was added to the sample cuvette. There was an immediate oxidation of cytochrome *b*, with little effect on cytochromes *a* or *c*. Indeed, there appeared to be a slight reduction of cytochrome *c*. In contrast the addition of ferricyanide led to the oxidation of cytochromes *a*, *b* and *c*. It appeared that Fe^{3+} was reduced by some electron-transport carrier that is in equilibrium with cytochrome *b*.

Other experiments to localize sites of iron reduction gave essentially negative results. Flight-muscle mitochondria of blowflies contain a very active α -glycerophosphate dehydrogenase that is located on the outside of the inner membrane (Klingenberg, 1970). The oxidation of α -glycerophosphate by a preparation of flight-muscle mitochondria was inhibited by antimycin A, but the addition of FeCl_3 did not result in the resumption of O_2 uptake.

Table 1. *Availability of iron from various complexes for reduction by whole and sonicated rat liver mitochondria and for ferrochelatase of sonicated mitochondria*

Rates of O₂ uptake were used to measure Fe²⁺ production (see Fig. 4) with succinate as electron donor, and ferrochelatase was assayed as described (see the Experimental section and Fig. 1) with NADH as electron donor. Iron compounds were added to give a final concentration of 1 mM-iron, except for transferrin and ferredoxin, which were 0.2 mM with respect to iron. Because rates were rarely linear, results for O₂ uptake are expressed only on a comparative basis. + indicates a maximum rate around 15 ng-atoms of O/min per mg of protein; +++ indicates a maximum rate around 50 ng-atoms of O/min per mg of protein.

Iron compound	Oxygen uptake		Ferrochelatase in sonicates (nmol of deuterohaem/min per mg of protein)
	in mitochondria	in sonicates	
FeCl ₃	*	+++	0.7
Ferrioxamine G	+++	+++	0.5
Ferrioxamine B	*	+	0.5
Ferrioxamine D	—	—	—
Ferrirubin	—	*	—
Ferrichrome	—	—	—
Ferrichrome A	—	—	—
Ferrichrysin	—	—	—
Ferrocene	—	—	—
Imferron	—	—	*
Transferrin	—	—	—
Ferritin	—	—	—
Ferredoxin	—	—	—

* Indicates that activity was so low that accurate rates could not be measured.

Discussion

The study of the metabolism of iron at physiological pH values presents many difficulties arising from the low solubility of iron salts and from the ready autoxidizability of Fe²⁺. Our results show that this latter property may be used in measuring the rate of reduction of added Fe³⁺. The rates of O₂ uptake noted when the Fe²⁺, formed by mitochondrial reduction of Fe³⁺ or ferrioxamine G, was allowed to autoxidize, were equal to or greater than the rates of O₂ uptake when mitochondria or electron-transport particles were oxidizing substrates by normal electron transport (Fig. 4). The poor solubility of the added iron salts may explain the requirement for fairly high concentrations of added iron (Figs. 6 and 7) and the apparent failure of added iron to continue through repeated cycles of oxidation and reduction changes (Fig. 5). By assaying the rates of haem synthesis catalysed by ferrochelatase, an enzyme that is specific for reduced iron, it was possible to show that the Fe²⁺ produced by mitochondrial activity persisted for sufficient time to satisfy any requirements for haem synthesis. Even under aerobic conditions there was evidence of a low but definite production of Fe²⁺ (Fig. 1). Since the normal environment of the mitochondria must be aerobic, this aerobic rate may reflect the normal potential of the

mitochondrion to synthesize haem and its capacity to maintain a concentration of Fe²⁺ around the *K_m* for ferrochelatase, somewhere between 12 μM (Labbe & Hubbard, 1961) and 60 μM (Yoneyama *et al.*, 1965). This supply of Fe²⁺ is important, not only for the maintenance of haem synthesis, but also for the functioning of the tricarboxylate cycle; the enzyme aconitase has a requirement for Fe²⁺ (Morrison, 1954) and, like ferrochelatase, is located within the mitochondrial matrix. Our experiments with intact mitochondria (Fig. 4b) suggest that Fe³⁺ is not reduced on the outside of the mitochondrial membrane. Potassium ferricyanide is well known to interact with electron-transport carriers on the outer surface of the inner mitochondrial membrane (see, e.g., Klingenberg, 1970) and it is perhaps surprising that Fe³⁺ is not reduced at the same site. The comparison of rates of reduction of Fe³⁺ by whole mitochondria and by mitochondrial sonicates (Figs. 4a and 4b) strongly suggests that iron reduction takes place inside the mitochondria, and the lack of activity of the soluble matrix fraction (Table 2) indicates that iron reduction is a property of the inner face of the mitochondrial membrane.

The measurement of the restoration of O₂ uptake by intact mitochondria inhibited with antimycin A or rotenone has made it possible to measure the

permeability of mitochondria to complexes of iron. The list of compounds that we have examined (Table 1) is obviously not exhaustive. Nevertheless, the

interesting observation that only ferrioxamine G (in contrast to the closely related compounds ferrioxamines B and D₁) readily penetrated the mitochondria, could provide useful chemical information

Fig. 6. Effect of Fe³⁺ and ferrioxamines B and G on (a) the time required to reach maximum rate of haem formation, and (b) the maximum rate of haem formation by sonicated mitochondria

Ferrochelatase activity was determined as stated in the Experimental section. The reaction mixture contained 2.5 ml of tris-HCl buffer (0.1 M), pH 7.2, containing 0.1% (v/v) Tween 80, sonicated mitochondria (8 mg of protein), NADH (2.5 μ mol) and deuteroporphyrin (0.1 μ mol). The reaction was started by addition of the iron compound to the concentration indicated, and the $\Delta(E_{498} - E_{511})$ was recorded. ●, Ferrioxamine B; ○, ferrioxamine G; □, Fe³⁺.

Fig. 7. Effect of Fe³⁺ and ferrioxamine G concentrations on (a) maximum rate of O₂ uptake and (b) time taken to reach maximum activity in sonicated mitochondria

O₂ uptake was used as a measure of formation of Fe²⁺. The incubation medium in the oxygen-electrode vessel contained KCl (80 mM), tris-HCl buffer (20 mM), pH 7.2, sonicated mitochondria (8 mg of protein), succinate (4 mM) and antimycin A (10 μ g). The reaction was started by the addition of Fe³⁺ (□) or ferrioxamine G (○) to the concentration shown. The final volume was 5.5 ml and temperature was 36°C.

Table 2. *Distribution of NADH-linked and succinate-linked iron-reducing sites in mitochondria*

Haem formation, determined as described in Fig. 1, was used as a measure of production of available Fe^{2+} . The respiratory substrates were added to give 1 mM final concentration. Particulate and soluble fractions of mitochondrial sonicates were separated by centrifugation at 100000g for 80 min. Particles were resuspended in the original volume of medium (250 mM-sucrose, 4 mM-tris-HCl buffer, pH 7.2).

Substrate	Mitochondrial fraction	Ferrochelatase (nmol of deuterohaem/min per mg of protein)	Ferrochelatase recovery (%)
Citrate+NAD ⁺	Whole sonicate	0.462	100
	Particles	0.0	0
	Particles+soluble	0.45	97
	Soluble	0.012	3
	Soluble+particles	0.438	95
NADH	Whole sonicate	0.587	100
	Particles	0.425	73
	Soluble	0.0	0
	Soluble+particles	0.462	79
Succinate	Whole sonicate	0.362	100
	Particles	0.225	62
	Soluble	0.0	0
	Soluble+particles	0.262	72

Fig. 8. *Effect of oxidizable substrate concentration on haem formation by sonicated mitochondria*

Ferrochelatase activity was determined as described in the Experimental section. Sonicated mitochondria (8 mg of protein) were incubated in 2.5 ml of 0.1 M-tris-HCl buffer, pH 7.2, containing 0.1% (v/v) Tween 80, deuteroporphyrin (0.1 μ mol), FeCl_3 (1.0 μ mol) and either NADH (○) or succinate (●) as indicated. Activities obtained with mixtures of NADH+succinate are indicated by □. For such mixtures the concentration of NADH is shown in parentheses and the concentration of succinate is that given on the abscissa.

about iron transport. The latter compounds differ from ferrioxamine G in minor substituents, but only ferrioxamine G is amphoteric; it has both a $-\text{CO}_2\text{H}$

Fig. 9. *Spectroscopic changes after (a) dithionite reduction of ferrioxamine B and (b) the enzymic incorporation of Fe^{2+} into deuteroporphyrin*

(a) Ferrioxamine B (1 mM) was incubated in a 2 mm light-path cuvette in a conventional spectrophotometer at 25°C. Spectra were recorded before and after the addition of dithionite. (b) Sonicated mitochondria (8 mg of protein), deuteroporphyrin (300 μ mol) and NADH (2.5 μ mol) were incubated at 37°C in 2.5 ml of 10 mM-tris-HCl buffer, pH 7.2, containing 0.1% (v/v) Tween 80 in a 1 cm light-path cuvette. Spectra were recorded before addition of Fe^{2+} (0.5 μ mol) and at 6, 12, 18 and 36 min after the addition. Arrows indicate the directions of the changes in extinctions.

Fig. 10. Reduction of ferrioxamine B and incorporation of Fe^{2+} into deuterohaem

Sonicated mitochondria (8mg of protein), NADH ($2.5\mu\text{mol}$) and deuteroporphyrin ($100\mu\text{mol}$) were incubated in 2.5ml of 100mM-tris-HCl buffer, pH7.2, containing 0.1% (v/v) Tween 80 for 3min (measured from anaerobiosis). The reaction was started by the addition of ferrioxamine B ($2.5\mu\text{mol}$). The change in extinction was recorded either at 498 minus 511nm (for haem formation) or 480.4 minus 581.4nm (for ferrioxamine B reduction). For reduction of ferrioxamine B $\Delta E_{\text{nm}} = 1.275$ was used and for metalloporphyrin formation $\Delta E_{\text{nm}} = 10$ was used. (a) Reduction of ferrioxamine B in the presence of deuteroporphyrin; (b) appearance of deuterohaem; (c) reduction of ferrioxamine B in the absence of porphyrin.

and an $-\text{NH}_2$ substituent and could possibly be accommodated on an amino acid permease of the membrane (Fig. 12). Although added Fe^{3+} ions appear to cause some deterioration of mitochondria, the presence of ferrioxamine G did not result in a loss of respiratory control (Fig. 5b), and further, it is unlikely that results can be explained by some disruption of mitochondrial integrity. Carrier systems for the transport of citrate are known to function across mitochondrial membranes (Chappell, 1968). Since citrate stabilized Fe^{3+} in solution it may be important in the cellular metabolism of iron. Addition of citrate did not, however, increase the rate of uptake of Fe^{3+} by intact mitochondria.

Only ferrioxamines G and B were readily reduced by mitochondrial particles and the other microbial

Fig. 11. Effect of the addition of Fe^{3+} on the redox state of cytochromes of sonicated mitochondria

Sonicated mitochondria (16mg of protein), NADH ($5\mu\text{mol}$), glutamate ($5\mu\text{mol}$) and antimycin A ($10\mu\text{g}$) were incubated in 5.0ml of 0.1M-tris-HCl buffer, pH7.2, containing 0.1% (v/v) Tween 80, at 26°C for 2min. Then $5\mu\text{g}$ of rotenone was added and the mixture was divided equally between two cuvettes. The split-beam base line was recorded as shown (a). Ferric citrate ($1.0\mu\text{mol}$) was added to the reference cuvette and the difference spectrum was recorded (b).

iron carriers were ineffective, as were the mammalian iron complexes ferritin and transferrin. Although transferrin may release its iron after reduction (Miller & Perkins, 1969) it is possible that the reduction mechanism involved is different from that for the mitochondria; it has also been shown that injected ^{59}Fe is not incorporated into haem via ferritin (Primossigh & Thomas, 1968). Ferredoxin was included in our tests as a possible analogue of the non-haem-iron proteins of mitochondria, although it has, of course, much lower mid-point potential. From our negative results we would confirm that some other form of iron complex serves as the donor of iron for haem synthesis in mammals.

The reduction of added ferrioxamine G was not inhibited by antimycin A or rotenone with NADH-linked substrates, and reduction of FeCl_3 was not inhibited by antimycin A when succinate was a substrate. When these iron compounds were added to mitochondria or particles respiring in the absence of inhibitor their effects on O_2 uptake were very slight and not additive. This suggests that the iron compounds are reduced by the respiratory chain and not by some special independent pathway. There must be at least two contributing paths of iron reduction, i.e. between succinate and the antimycin A block and between NADH and the rotenone block. Spectroscopic evidence that one site of iron reduction is in equilibrium with cytochrome *b* is provided in Fig. 11,

Fig. 12. Structures of some of the iron complexes used in this study.

which shows that the addition of Fe^{3+} to anaerobic mitochondrial particles is followed by an oxidation of cytochrome *b*, when suitable inhibitors are present to isolate this region of the respiratory chain. The slight reduction of cytochrome *c* that is apparent may be due to the reducing activity of the newly formed Fe^{2+} . Possible sites for the interactions of Fe^{3+} with the respiratory chain are the flavoproteins NADH dehydrogenase and succinate dehydrogenase and we suggest a possible localization for these reactions in Scheme 2. Ferrous iron is produced on the inner surface of the inner mitochondrial membrane, where ferrochelatase is also located. The first enzyme

of haem biosynthesis, δ -aminolaevulinatase synthetase, is located within the matrix space and this enzyme may be inhibited if the free haem concentration increases (Scholnick *et al.*, 1969). The structural organization of the reactions of haem synthesis that we propose is such as to promote a regulated synthesis of haem, but problems of the subsequent interaction of haem with various apoproteins remain to be solved.

When ferric iron was added to mitochondrial sonicates there was a lag period before the maximum rate of haem synthesis developed, even when the particles were allowed to become anaerobic after

Scheme 2. *Proposed organization of the enzymes and permeases of the inner mitochondrial membrane involved in the reduction of Fe^{3+} and utilization of the Fe^{2+} formed*

Cyt. indicates cytochrome. X indicates a postulated intermediate (see the text).

the addition of NADH or succinate. It appeared likely that this lag was due to the time taken to accumulate concentrations of Fe^{2+} approaching the K_m for ferrochelatase, but direct measurements of the rate of reduction of the iron of ferrioxamine B (Fig. 10) also showed a lag period,

correlating well with the lag period of haem synthesis. Measurements of the extent of reduction of cytochromes after the anaerobic change were useful in suggesting an alternative explanation for the lag. Cytochromes *a* and *c* (measured at 605 minus 360nm and 550 minus 540nm respectively) became 95%

reduced within 30s; cytochrome *b* (measured at 560 minus 575 nm) took appreciably longer to reach 95% reduction (180s). Thus the electron-transport carrier that couples with the Fe^{3+} may not become fully reduced for several minutes after the mitochondria have exhibited the anaerobic change, and this delay may partly explain the lag period. The absence of additive rates of Fe^{3+} reduction when both NADH and succinate were added as substrates (Fig. 8) has led us to postulate an intermediate 'X' between the flavoprotein dehydrogenases and the Fe^{3+} . This intermediate 'X' becomes fully saturated by NADH under anaerobic conditions, and so added succinate does not increase rates of Fe^{3+} reduction. The observation that ferrioxamine B reduction is greatly stimulated when porphyrin is added to permit haem formation (Fig. 10) suggests that iron reduction may only persist when Fe^{2+} is utilized. This would have great metabolic significance in preventing a continual drain of electrons from the respiratory chain.

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