Two types of receptors for iron on mitochondria

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ATP, the major ligand for Fe in the reticulocyte's low- M_r chelatable pool, transfers Fe to a mitochondrial receptor from which the Fe is incorporated into haem. However, the utilization of Fe bound to this receptor for haem synthesis is slow in comparison with that bound to a second receptor that does not accept Fe from ATP, but does accept it from AMP. The major pathway by which Fe is delivered for haem synthesis may be through hydrolysis of cytosolic ATP-Fe to AMP-Fe.

INTRODUCTION

The means by which intracellular iron is made available to mitochondria for haem synthesis is not clear. Mitochondria can take up Fe from unliganded Fe(II) and Fe(III) in an anaerobic environment (Barnes *et al.*, 1972). However, it is unlikely that significant concentrations of either free Fe(II) or Fe(III) are present in the reticulocyte cytosol; Fe(II) would be oxidized and Fe(III) would be precipitated. Also, when reticulocytes take up Fe from transferrin while their haem-synthesis pathway is blocked, no detectable Fe(II) or Fe(III) accumulates in the cytosol (n.m.r. was used to measure ^{31}P relaxation, a phenomenon strongly influenced by the concentration of metal in the neighbourhood of phosphate) (Zhan *et al.*, 1990a).

Mitochondria can take up Fe from ferritin, but Fe(II) must be first released from the ferritin. Moreover, the argument that ferritin is a significant donor because there are mitochondrial ferritin receptors (Ulvik & Romslo, 1978, 1979; Ulvik, 1982) has been weakened by the failure of Funk *et al.* (1986) to find ferritin receptors. Mitochondria can take up Fe from transferrin, but in amounts too small to be significant (Romslo, 1980).

Mitochondrial uptake of Fe from phosphate-containing compounds has been studied for several years (Konopka & Romslo, 1980, 1981; Nilsen & Romslo, 1984a,b). The recent finding that, when ATP-Fe is incubated with mitochondria, Fe binds with high affinity to a mitochondrial receptor (Weaver *et al.*, 1990), at first suggested that ATP might be the important physiological intracellular donor of Fe for haem synthesis. The present work suggests, instead, that ATP is the penultimate donor, and that the major pathway by which Fe is delivered for haem synthesis is through hydrolysis of cytosolic ATP-Fe to AMP-Fe.

MATERIALS AND METHODS

ATP, AMP and Hepes were obtained from Sigma; $^{59}\text{FeCl}_3$, [^{14}C]AMP and NCS tissue solubilizer were obtained from Amersham; Aquasol II was from New England Nuclear, and deuteroporphyrin IX hydrochloride was from Porphyrin Products, Logan, UT, U.S.A. All reagents were of the highest purity available and were not further purified. Deionized glass-distilled water was used throughout.

Preparation of mitochondria

The procedure followed was that of Johnson & Lardy (1967). Non-fasting Sprague-Dawley rats, average weight 279 g, were stunned and their livers rapidly removed and minced; 5 g of liver

was homogenized in 12.5 ml of 250 mM-sucrose/10 mM-Hepes, pH 7.4 (sucrose/Hepes) and diluted to 40 ml with the same buffer (0 °C). The homogenate was centrifuged at 600 *g* for 10 min, the supernatant was collected and the pellet resuspended in 10 ml of sucrose/Hepes, centrifuged again at 600 *g* for 10 min and the second supernatant was collected. The combined supernatants were then centrifuged at 15000 *g* for 5 min, the resulting supernatant discarded, and the pellet resuspended in 225 mM-sucrose/10 mM-KCl/5 mM-succinate/10 mM-Hepes, pH 7.4 ('Buffer'). Protein concentration in the mitochondrial suspension was determined by the Biuret method (Weichselbaum, 1946).

Preparation of nucleotide-Fe complexes

Complexes were freshly prepared each day. The nucleotide was dissolved in Buffer and the pH adjusted to 7.4; then the appropriate amount of FeCl_3 in 0.5 M-HCl, containing ^{59}Fe (5 $\mu\text{Ci}/\text{ml}$ in the final solution) was added slowly with constant stirring while the pH was maintained by addition of aliquots of 0.5 M-NaOH.

Mitochondrial Fe uptake at 0 °C

As previously described (Weaver *et al.*, 1990), mitochondrial suspension (40–60 mg/ml) was mixed with Buffer containing nucleotide- ^{59}Fe (0.1 ml of mitochondrial suspension/ml of final suspension), kept in ice for a designated time; then centrifuged at 15000 *g* for 5 min; the pellet was washed three times with Buffer and the radioactivity measured in a Tracor γ -radiation counter.

Deuterohaem synthesis by mitochondria

Unless otherwise designated, ^{59}Fe -labelled mitochondria were prepared as described above. Duplicate portions of the labelled mitochondria were resuspended in Buffer (0.1 ml of mitochondrial suspension in 0.5 ml of Buffer) which had been warmed to 30 °C and which contained 20 μM -deuteroporphyrin. Incubation at 30 °C was continued for the designated time; at the conclusion of the incubation, samples were placed on ice. A 0.05 ml portion of 0.1 M- $\text{K}_3\text{Fe}(\text{CN})_6$ was added to each 0.5 ml of suspension and the sample was left for 10 min; then 0.15 ml of 1.0 M-HCl was added, and the sample left for 10 min; then 1.0 ml of butan-2-one (0 °C) was added and mixture shaken vigorously. Two phases separated; the ^{59}Fe in the organic phase was measured. There was negligible radioactivity in the organic phase when mitochondria were incubated without deuteroporphyrin, or when mitochondria were kept at 0 °C; ^{59}Fe

Abbreviation used: Fe, unspecified Fe(II) or Fe(III).

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appearance in the organic phase was linear with time when mitochondria were incubated at 30 °C with ATP-⁵⁹Fe or AMP-⁵⁹Fe.

Mitochondrial [¹⁴C]AMP uptake

Portions of mitochondrial preparations (0.1 ml/ml of sample) were incubated at 0 °C for 60 min in Buffer containing 600 μM-AMP as [¹⁴C]AMP-Fe (AMP/Fe, 40:1), [¹⁴C]AMP, or AMP-⁵⁹Fe (AMP/Fe, 40:1). Samples were washed three times in Buffer (0 °C), dissolved in tissue solubilizer, suspended in Aquasol II, and the radioactivity was measured in an LKB 1215 Rackbeta II liquid-scintillation counter; ⁵⁹Fe samples were washed three times and counted for radioactivity in the γ-radiation counter.

RESULTS

Earlier work showed that, when ATP-Fe was incubated with mitochondria, Fe, free of ATP, bound with high affinity to receptors. The binding was saturable and reversible. In contrast, Fe from AMP-Fe bound to mitochondria non-specifically (Weaver *et al.*, 1990). Mitochondria were loaded with specifically bound Fe by incubating them with ATP-Fe (4:1 complex; 60 μM-Fe) and with non-specifically bound Fe by incubating them with AMP-Fe (4:1 complex, 60 μM-Fe) (both 0 °C, 30 min). They were then warmed to 30 °C, and Fe incorporation into deuterohaem was measured. The non-specifically bound Fe was

Fig. 1. Deuterohaem synthesis by mitochondria from Fe taken up at 0 °C from ATP-Fe (4:1) and AMP-Fe (4:1)

Rat liver mitochondria suspended in 225 mM-sucrose/10 mM-KCl/5 mM-succinate/10 mM-Hepes, pH 7.4, were incubated with ATP-⁵⁹Fe or AMP-⁵⁹Fe (nucleotide/Fe, 4:1; 60 μM-Fe), freshly prepared in the same buffer, at 0 °C for 30 min. After incubation, samples were centrifuged at 15000 g for 5 min, washed three times in the same buffer at 0 °C. They were then resuspended in the same buffer, which had been warmed to 30 °C and which contained 20 μM-deuteroporphyrin; incubation at 30 °C was continued for the designated time, after which the sample was placed on ice and deuterohaem was extracted. Results of an average of six experiments (±SD) are shown. Fe uptake was 2.16 (±1.04) nmol/mg of protein from AMP-Fe and 0.402 (±0.103) nmol/mg of protein from ATP-Fe. Incorporation of Fe into haem from Fe contributed by AMP-Fe was significantly greater than that from Fe contributed by ATP-Fe at all time periods (*P* < 0.05; two-tailed *t*-test). The inset shows the percentage of bound Fe utilized for haem synthesis; the percentage incorporation of Fe into haem from Fe contributed by AMP-Fe was significantly greater than that contributed by ATP-Fe at 10 and 20 min (*P* < 0.05; one-tailed *t* test). ○, Deuterohaem synthesis from ATP-Fe; △, deuterohaem synthesis from AMP-Fe.

Fig. 2. Uptake of Fe by mitochondria from AMP-Fe at various AMP/Fe ratios

Rat liver mitochondria, as described in the legend to Fig. 1, were mixed with the designated concentrations of Fe(III) in complexes with ATP or AMP. After incubation at 0 °C for 60 min, samples were centrifuged at 15000 g for 5 min, washed three times at 0 °C, and the pellet was counted for radioactivity. The figure shows average results for two independent experiments. Symbols: ●—●, ATP-Fe (4:1); ▲---▲, AMP-Fe (4:1); ▲.....▲, AMP-Fe (40:1); ▲.....▲, AMP-Fe (100:1); ▲——▲, AMP-Fe (400:1).

utilized for deuterohaem synthesis better than the specifically bound Fe (Fig. 1).

AMP binds Fe weakly; thus polynuclear Fe formation, when the ligand:Fe ratio is 4:1, is likely, and could account for the extensive non-specific binding of Fe to mitochondria which, in turn, could mask a component of saturable binding to a receptor. Solutions with various AMP/Fe ratios were prepared and incubated with mitochondria at 0 °C. Those in which the ratio of AMP:Fe was 40:1 or greater showed saturable binding of the Fe to mitochondria (Fig. 2). When binding of the AMP-⁵⁹Fe (40:1 complex) was studied in the presence of a 200-fold excess of unlabelled AMP-Fe (40:1), 98 % of the ⁵⁹Fe was displaced. When mitochondria which had been incubated (0 °C) with AMP-⁵⁹Fe (40:1) were washed and reincubated with a 200-fold molar excess of unlabelled AMP-Fe (40:1), 64 % of the Fe exchanged (average of results for two experiments). Thus ⁵⁹Fe, presented to mitochondria as AMP-Fe, exhibited saturable and reversible binding.

We attempted to determine whether Fe bound free of AMP: mitochondria were incubated at 0 °C for 60 min in Buffer containing either 600 μM-[¹⁴C]AMP-Fe (40:1), [¹⁴C]AMP or AMP-⁵⁹Fe (40:1). Uptake of [¹⁴C]AMP was 5.00 (±1.03) nmol/mg of protein: of [¹⁴C]AMP from AMP-Fe (40:1), 5.24 (±0.92) nmol/mg of protein; of Fe from AMP-⁵⁹Fe, 0.38 (±0.097) nmol/mg of protein (average of five independent experiments); the difference between AMP binding with and without Fe was not statistically significant. However, the increment in AMP binding, were the Fe binding as an AMP-Fe complex, would have been less than one-tenth the amount of AMP which bound when no Fe was present (i.e. less than 1 s.d. of the AMP binding). Thus it is not possible to decide from these measurements whether or not Fe binds free of AMP. It may be noted that AMP binding to mitochondria differs from that of ATP, where only 0.002–0.005 nmol of ATP was bound/mg of protein.

At saturation, the amount of Fe taken up from ATP-Fe (4:1) and from AMP-Fe (40:1) was almost the same, suggesting that the same receptor might be used. However, there was additive

Fig. 3. Mitochondrial Fe uptake from ATP-Fe (4:1) and from AMP-Fe (40:1), followed by Fe uptake from the other nucleotide-Fe complex

Rat liver mitochondria, as described in Fig. 2, were incubated with $60 \mu\text{M}$ -Fe(III) in complexes with ATP (4:1) or AMP (40:1) (0°C for 30 min) and washed. One set of pellets was counted for radioactivity, and a second was reincubated at 0°C for 30 min with $60 \mu\text{M}$ -Fe(III) in complex with the other nucleotide, then washed and counted for radioactivity. Average results from two independent experiments are shown.

Fig. 4. Deuterohaem synthesis by mitochondria from Fe taken up at 0°C from ATP-Fe (4:1) and AMP-Fe (40:1)

The experiment was performed as described in Fig. 1, using AMP-Fe (40:1) ($60 \mu\text{M}$ -Fe) in place of ATP-Fe (4:1). Fe uptake was 0.45 nmol/mg of protein from ATP-Fe and 0.42 nmol/mg of protein from AMP-Fe. The Figure shows average results for two independent experiments. $\circ$, Deuterohaem synthesis from ATP-Fe; Δ , deuterohaem synthesis from AMP-Fe.

binding when mitochondria were incubated with a saturating amount of ATP-Fe (4:1) or AMP-Fe (40:1), washed, and reincubated with a saturating amount of the other nucleotide-Fe complex, suggesting that two sets of receptors are present: one accessible to ATP-Fe and the other to AMP-Fe (Fig. 3).

The efficiency with which Fe was incorporated into deuterohaem when each of the two types of receptors were loaded at 0°C with similar amounts of Fe was studied. The receptors which received Fe from AMP-Fe were more efficient donors of Fe for deuterohaem synthesis (Fig. 4).

When the incubation of mitochondria with ATP-Fe (4:1) or AMP-Fe (40:1) was carried out at 30°C , with samples removed at intervals and analysed for Fe uptake and deuterohaem synthesis, ATP proved to be at least equal to AMP as an Fe donor for deuterohaem synthesis (Fig. 5), suggesting that, at 30°C , hydrolysis of the ATP-Fe generates AMP-Fe.

Fig. 5. Deuterohaem synthesis by mitochondria from Fe taken up at 30°C from ATP-Fe (4:1) and AMP-Fe (40:1)

Rat liver mitochondria suspended in 225 mM -sucrose/ 10 mM -KCl/ 5 mM -succinate/ $20 \mu\text{M}$ -deuteroporphyrin/ 10 mM -Hepes, pH 7.4, were incubated with ATP- ^{59}Fe (ATP/Fe, 4:1; 60 mM -Fe) or AMP- ^{59}Fe (AMP/Fe, 40:1; $60 \mu\text{M}$ -Fe) at 30°C . At the designated times, two sets of samples were taken from the incubation mixture. One set was centrifuged at 15000 g , washed three times in the same buffer at 0°C , and the pellets counted for radioactivity; deuterohaem was extracted from the other set. Average results for three independent experiments are shown. $\bullet$, Fe taken up from ATP-Fe; $\blacktriangle$, Fe taken up from AMP-Fe; $\circ$, deuterohaem synthesis from ATP-Fe; Δ , deuterohaem synthesis from AMP-Fe.

DISCUSSION

There appear to be two kinds of mitochondrial receptors for Fe: one that accepts Fe from ATP (and not from AMP) and which provides Fe for haem synthesis with relatively low efficiency, and the other that accepts Fe from AMP (and not from ATP) and which provides Fe for haem synthesis with relatively high efficiency. Since ATP is the principal ligand for Fe in the cytosol (Weaver & Pollack, 1989), the major route by which Fe is delivered for haem synthesis is probably by hydrolysis of cytosolic ATP-Fe to AMP-Fe (a process probably facilitated by the Fe) (Jones *et al.*, 1983). AMP-Fe has been consistently found in reticulocyte cytosol, despite varying experimental conditions (Pollack *et al.*, 1985; Weaver & Pollack, 1989). Cytosolic AMP-Fe probably reflects a balance between competing reactions: production by hydrolysis of ATP-Fe, and depletion by mitochondrial uptake and by recombination with intact ATP.

The apparent independence of the two Fe receptors merits some speculation. A relatively facile explanation for the failure of Fe in the ATP-Fe complex to transfer to the AMP-Fe receptor is possible: the iron may be bound too tightly by ATP. The explanation of the failure of AMP-Fe to transfer Fe to the high-affinity ATP-Fe receptor is more laboured: (1) AMP-Fe may not bind to the ATP-Fe receptor because the AMP-Fe complex may be too large for the Fe to reach its binding site in the ATP-Fe receptor. AMP- Fe_1 ($M_r > 2000$) is probably the dominant species in solution, because the AMP phosphate oxygen atoms are more likely to be electron donors to Fe than the adenine nitrogen atoms and, with the huge excess of phosphate,

there is probably no chelation in the AMP solution but, rather, a cluster of AMP around each Fe atom. ATP-Fe, on the other hand, is $\text{ATP}_1\text{-Fe}_1$ or $\text{ATP}_2\text{-Fe}_1$ (M_r about 600–1200) (Goucher & Taylor, 1964). (2) The ligand may effect a conformational change in the receptor which exposes an otherwise covered site. (3) The Fe bound to the AMP-Fe receptor may be bound as AMP-Fe rather than as Fe.

Earlier work studying the effect of various phosphates on Fe delivery to mitochondria for haem synthesis (Konopka & Romslo, 1980, 1981; Nilsen & Romslo, 1984a,b) used transferrin as a donor reservoir for Fe and measured Fe incorporation into haem. Thus a complex reaction which, in retrospect, probably included Fe release from transferrin, hydrolysis of the ligand-Fe complex, receptor binding and haem synthesis, was subsumed in a single measurement. The conclusion suggested by those studies, that pyrophosphate might be the physiological ligand for transferring Fe to mitochondria, left unanswered the question of how pyrophosphate could serve this function when so little is detectable (Yamada *et al.*, 1988); also, there is no detectable pyrophosphate-Fe in the cytosol of the reticulocyte (Weaver *et al.*, 1990). More recent work suggests that ATP may be the agent which removes Fe from endocytosed transferrin (Nunez *et al.*, 1989) and that ATP binds Fe in the cytosol (Weaver & Pollack, 1989).

What might be the function of the receptor which accepts Fe only from ATP-Fe and delivers Fe for haem synthesis relatively slowly? It might supply Fe to the mitochondrial non-haem Fe pool; it might be part of an accessory pathway for haem synthesis (we have recently found two pathways for removal of Fe from intracellular transferrin; Zhan *et al.*, 1990b); it might serve to

remove Fe from low- M_r ligands in the cytosol to keep levels below a toxic threshold.

REFERENCES

- Barnes, R., Connelly, J. L. & Jones, O. T. G. (1972) *Biochem. J.* **128**, 1043–1055
- Funk, F., Lecrenier, C., Lesuisse, E., Crichton, R. R. & Schneider, W. (1986) *Eur. J. Biochem.* **157**, 303–309
- Goucher, C. R. & Taylor, J. F. (1964) *J. Biol. Chem.* **239**, 2251–2255
- Johnson, D. & Lardy, H. (1967) *Methods Enzymol.* **10**, 94–96
- Jones, D. R., Londoy, L. F. & Sargeson, A. M. (1983) *J. Am. Chem. Soc.* **105**, 7327–7336
- Konopka, K. & Romslo, I. (1980) *Eur. J. Biochem.* **107**, 433–439
- Konopka, K. & Romslo, I. (1981) *Eur. J. Biochem.* **117**, 239–244
- Nilsen, T. & Romslo, I. (1984a) *Biochim. Biophys. Acta* **766**, 233–239
- Nilsen, T. & Romslo, I. (1984b) *Biochim. Biophys. Acta* **802**, 448–453
- Nunez, M. T., Watkins, J. A., Gaete, V., Li, C. Y., Elder, P. & Glass, J. (1989) *Blood* **74** (Suppl. 1) 61a
- Pollack, S., Campana, T. & Weaver, J. (1985) *Am. J. Hematol.* **19**, 75–84
- Romslo, I. (1980) in *Iron in Biochemistry and Medicine*, vol. 2 (Jacobs, A. & Worwood, M., eds.), pp. 325–362, Academic Press, London
- Ulvik, R. & Romslo, I. (1978) *Biochim. Biophys. Acta* **541**, 251–262
- Ulvik, R. & Romslo, I. (1979) *Biochim. Biophys. Acta* **588**, 256–271
- Ulvik, R. J. (1982) *Biochim. Biophys. Acta* **715**, 42–51
- Weaver, J. & Pollack, S. (1989) *Biochem. J.* **261**, 787–792
- Weaver, J., Zhan, H. & Pollack, S. (1990) *Biochem. J.* **265**, 415–419
- Weichselbaum, T. E. (1946) *Am. J. Clin. Pathol.* **16**, 40–49
- Yamada, T., Inoue, T., Nishida, T., Furuya, E. & Tagawa, K. (1988) *J. Biochem. (Tokyo)* **104**, 647–850
- Zhan, H., Gupta, R. K., Weaver, J. & Pollack, S. (1990a) *Eur. J. Haematol.* **44**, 125–131
- Zhan, H., Pollack, S. & Weaver, J. (1990b) *Eur. J. Haematol.*, in the press

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