Mitochondria have Fe(III) receptors

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Recent work has provided new evidence that ATP is the major constituent of the low- M_r iron pool in the reticulocyte. The interaction of the iron complex of ATP with mitochondria was investigated in the present experiments. When ATP-Fe³⁺ was incubated with mitochondria, Fe³⁺, free of ATP, bound with high affinity to Fe³⁺ receptors on the mitochondria. The binding was saturable and reversible. Iron which was complexed to PP_i, nitrilotriacetate, citrate, ADP and GTP also showed saturable binding to mitochondria; Fe³⁺ complexed to AMP bound non-specifically, as did Fe²⁺/ascorbate and Fe²⁺/dithionite.

INTRODUCTION

When an animal is dosed with desferrioxamine (DF), a variable amount of iron is mobilized and excreted in urine and bile as a DF-Fe complex (Keberle, 1964). Mulligan *et al.* (1986), quantifying this chelatable pool by measuring the Fe which could be extracted by DF from different tissues, found that the amount of chelatable Fe correlated with that which was ultrafiltratable, thus showing that the ligands which were binding chelatable Fe were of low M_r . The nature of these ligands has been controversial. It has been suggested that ATP and GTP are the major constituents of the pool (Bartlett, 1970, 1976; Konopka & Szotor, 1972). But the early experiments supporting this suggestion involved acid extraction of haemoglobin-containing solutions, a procedure shown to liberate Fe from haem, with this released iron then binding to nucleotides (Meyers *et al.*, 1973).

Recent work, in which cytosolic proteins were precipitated with ammonium sulphate, thus avoiding acid extraction, has provided new evidence that ATP is the major constituent of the low- M_r Fe pool in the reticulocyte (Weaver & Pollack, 1989). The uptake of ATP-Fe by mitochondria has now been studied. When ATP-Fe³⁺ was incubated with mitochondria, Fe³⁺, free of ATP, bound with high affinity to Fe³⁺ receptors on the mitochondria. The binding was saturable and reversible. Fe complexed to PP_i, nitrilotriacetate, citrate, ADP and GTP also showed saturable binding to mitochondria; Fe³⁺ complexed by AMP bound non-specifically, as did Fe²⁺/ascorbate and Fe²⁺/dithionite.

MATERIALS AND METHODS

Materials

ATP (containing less than 0.052 μ mol of Fe/mmol), ADP, AMP, GTP, D-glycerate-2,3-bisphosphate (2,3-DPG), acetohydroxamic acid and Hepes were obtained from Sigma; ⁵⁹FeCl₃, [¹⁴C]ATP and NCS tissue solubilizer were obtained from Amersham; Aquasol II was obtained from New England Nuclear. 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 wt. 292 g, were stunned and their livers were rapidly removed and minced; 5 g of liver was homogenized in 12.5 ml of ice-cold 250 mM-sucrose/10 mM-Hepes, pH 7.4 (sucrose/Hepes). The homogenate was centrifuged at 600 g for 10 min, the supernatant was collected and the pellet was resuspended in sucrose/Hepes and centrifuged again at 600 g for 10 min. The second supernatant was collected. The combined supernatants were then centrifuged at 15000 g for 5 min, the resulting supernatant was discarded and the pellet was suspended in 225 mM-sucrose/10 mM-KCl/5 mM-succinate/10 mM-Hepes, pH 7.4 (buffer A).

Preparation of ligand-iron complexes and ferrous iron

Ligand/Fe solutions (4:1), at a final concentration of 2.4 mM-ligand, were always freshly prepared; preliminary experiments demonstrated that iron uptake from ATP-Fe solutions by mitochondria was saturable and reversible when fresh solutions were used but not consistently so when solutions were 48 h or more old. Ligand was dissolved in buffer A which was adjusted to pH 7.4; then FeCl₃ in 0.5 M-HCl, containing ⁵⁹FeCl₃ (5 μ Ci/ml in the final solution), was added slowly with constant stirring, the pH being maintained by addition of aliquots of 0.5 M-NaOH.

Ferrous iron was prepared immediately before use with different reductants. (1) FeSO₄ and ⁵⁹FeCl₃ in 0.01 M-HCl were added to 0.1 M-sodium dithionite in buffer A and the pH was re-adjusted to 7.4; the Fe concentration of the initial stock solution was 0.6 mM. Aliquots of the stock solution were added to buffer A containing 0.1 M-sodium dithionite to obtain the Fe concentrations designated in the experiments depicted. (2) Ferrous iron was also prepared in buffer A containing 60 mM-ascorbate with the other steps as described above.

In several experiments, the ligand-Fe solutions and the ferrous iron solutions were prepared in buffer A without succinate.

Mitochondrial iron uptake

In a typical experiment, 0.1 ml of mitochondrial suspension (30–46 mg of protein/ml) was added to 0.9 ml of

Abbreviations used: DF, desferrioxamine; 2,3-DPG, D-glycerate-2,3-bisphosphate.

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ligand-Fe/buffer A mixture; all reactants had been chilled on ice. The ligand/Fe ratio was 4:1 unless otherwise specified. The concentration of Fe, designated in individual experiments, ranged from 1.5 to 90 μM . Samples, always run in duplicate, were left on ice for 60 min, and then centrifuged (15000 g for 5 min); the pellet was then washed three times with ice-cold buffer A. The third wash contained less than 10% of the counts in the pellet. The ligand-Fe complexes studied formed stable solutions which adsorbed minimally to the polypropylene tubes utilized. Only 0.17% of the counts remained after the third wash in tubes run without mitochondria. This was a negligible amount except where Fe uptake by mitochondria was comparably small (i.e. in samples run against Fe-PP_i and Fe-nitritotriacetate), in which instances the appropriate corrections were made.

There was virtually identical binding of Fe to mitochondria when the ligand-Fe/Buffer A was made without succinate. Although succinate appeared to play little or no role in Fe binding to mitochondria at 0 °C, buffer A (containing succinate) was employed to minimize the departure from the conditions for mitochondrial incubation used in other studies (Nilsen & Romslo, 1985).

Mitochondrial [¹⁴C]ATP uptake

Aliquots of a single mitochondrial preparation were incubated as described above with the 4:1 complex of ATP-⁵⁹Fe, [¹⁴C]ATP-Fe and [¹⁴C]ATP. They were then washed as described above, solubilized in Amersham tissue solubilizer and suspended in Aquasol II, and radioactivity was counted in a liquid scintillation counter.

Estimation of relative affinity of ligands for Fe

The affinity for Fe of each ligand, relative to that of acetohydroxamate, was determined in competition experiments. The acetohydroxamate-Fe complex has an absorption maximum at 430 nm; when a competing ligand removes Fe from acetohydroxamate there is a decrease in absorbance at 430 nm. (The Fe complexes of the ligands tested in these experiments have no absorbance in this region.)

A 0.01 M-acetohydroxamate/0.2 mM-Fe solution in 0.1 M-NaCl/0.1 M-Hepes (pH 7.4) was prepared as described by Cowart *et al.* (1982). Acetohydroxamate-Fe solution (0.5 ml) was mixed with 1 ml of 0.1 M-NaCl/0.1 M-Hepes (pH 7.4) (the test solution thus contained 3.3 mM-acetohydroxamate/66.7 μM -Fe) which contained various concentrations of the ligand to be tested. The experiments were run at room temperature.

Control experiments established that the reaction between acetohydroxamate-Fe and the competing ligands was rapid; in most instances the removal of Fe from the acetohydroxamate was complete within minutes; the mixture then showed no change in absorbance at 430 nm for at least 48 h. ADP reacted more slowly; calculations for ADP were based on measurements made after 24 h of incubation.

The relative affinity of a ligand for Fe, compared with that of acetohydroxamate for Fe, is inversely proportional to the concentration of ligand required to remove a measured amount of Fe from the acetohydroxamate complex. That relative affinity was quantified by scoring against acetohydroxamate (with the latter given an arbitrary value of 1). The concentration of ligand needed to remove 30–70% of the

iron initially bound to acetohydroxamate ranged from 0.07 mM (PP_i) to 0.37 M (2,3-DPG).

The equations of binding may be represented as follows:

where AcOH = acetohydroxamate and L = ligand.

The relative affinity of the ligand for Fe (*R*) in comparison with that of acetohydroxamate for Fe is then:

$$R = [\text{FeL}] \cdot [\text{AcOH}] / [\text{FeAcOH}] \cdot [\text{L}] \quad (3)$$

Where the ligand contributes Fe, that contribution is represented as shown below, and the simultaneous eqns. (3) and (4) are solved to determine *R*.

$$\text{Total Fe} = \text{FeL} + \text{FeAcOH} \quad (4)$$

The composition of the ligand-Fe complex does not need to be specified to compute its relative affinity for Fe, since the mitochondrial uptake and acetohydroxamate competition experiments are both run with ligand excess.

Other procedures

Protein determination was with the Biuret method (Weichselbaum, 1946). Fe determination utilized the ferrozine method (Sigma).

RESULTS

Rat liver mitochondria were incubated at 0 °C with increasing concentrations of Fe in a 4:1 complex with ATP. Fe bound to the mitochondria. The binding was saturable: a 200-fold excess of unlabelled Fe in the initial incubation mixture displaced 97–99% of the bound counts (Fig. 1). The binding was also reversible: mitochondria saturated with Fe by incubation with the 4:1 ATP-Fe complex (60 μM -Fe) and reincubated (0 °C for 1 h) in fresh ATP-Fe lost 80% of their bound Fe.

Control experiments excluded the possibility that the binding of Fe by mitochondria could be explained by the insertion of Fe into transferrin-transferrin-receptor complexes which were in the mitochondrial pellet. Mitochondria were incubated with ⁵⁹Fe-labelled transferrin or with ATP-Fe and the results were compared (the incubation mixtures were equimolar in iron concentration). The transferrin-incubated samples bound less than 2% of the Fe bound by the ATP-Fe-incubated samples. Also, Fe taken up by the mitochondria from ATP-Fe did not exchange with transferrin (as would be anticipated if the Fe were bound to transferrin in a transferrin-transferrin-receptor complex). ATP did not bind to mitochondria together with the Fe (Table 1).

We investigated other Fe ligands to explore the requirements for donation of Fe to the mitochondria. When the Fe was presented bound to AMP, a ligand which binds Fe very weakly, binding was non-specific. Fe complexed by stronger ligands bound specifically. However, there seemed to be no other requirement: the adenine moiety was not required, as GTP was almost as efficient a donor as ATP; a base was not required, as 2,3-DPG was almost as efficient a donor as ATP; phosphate groups were not required as citrate was an efficient donor (Fig. 2).

The association constant for the Fe-mitochondria complex, with citrate as the competing ligand [calculating

Fig. 1. Binding of Fe to mitochondria

Inset: 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 (ATP/Fe, 4:1; 60 μM-Fe), freshly prepared in the same buffer, at 0 °C for the designated time. After incubation, samples were centrifuged at 15000 *g* for 5 min, washed three times in the same buffer at 0 °C and the pellet was counted for radioactivity. The result shown is a representative experiment. Main figure: rat liver mitochondria were incubated with varying concentrations of ATP-⁵⁹Fe at 0 °C for 60 min and then processed as described above. Specific binding was determined by incubation of duplicate samples to which unlabelled ATP-Fe (unlabelled Fe; ATP/Fe, 4:1; 18 mM-Fe) was added; the counts not displaced by the excess unlabelled ATP-Fe comprised the non-specific portion of the total counts bound. The results shown are the averages from three experiments. ●, Total binding, ○, specific binding.

Table 1. Binding of Fe and ATP to mitochondria

Mitochondria were incubated with ATP-Fe (4:1, 60 μM-Fe), with one group of samples containing ⁵⁹Fe and unlabelled ATP, the other group of samples containing [¹⁴C]ATP and unlabelled Fe. Incubations were at 0 °C for 1 h; all samples were in buffer A (pH 7.4). The Table shows the average of two independent determinations. Virtually identical amounts of ATP bound to the mitochondria whether presented as a 4:1 Fe complex or presented without iron.

[Fe] (μM)	Fe bound (μmol/g of protein)	ATP bound (μmol/g of protein)
1.5	0.13	0.003
3	0.21	0.005
6	0.34	0.003
12	0.50	0.003
24	0.79	0.002
36	0.88	0.004
60	1.02	0.003

free Fe as described by Aasa *et al.* (1963) and assuming that the mitochondrial Fe³⁺ receptor is in equilibrium with the free Fe in the system] was 10¹⁸ (Fig. 3). Fe complexed to ATP bound to the mitochondria receptor with an association constant of 10¹⁷ [using the calculation described by Aasa *et al.* (1963) and the relative affinity of

Fig. 2. Binding of Fe complexed with various ligands to mitochondria

Rat liver mitochondria, as described in the legend to Fig. 1, were mixed with varying concentrations of ferric iron in 4:1 complexes with AMP (●---●), ADP (●····●), GTP (●—●), 2,3-DPG (▲---▲), PP_i (▲—▲), citrate (■---■), nitrilotriacetate (■—■) or with ferrous iron in 0.1 M-sodium dithionite (□). Samples were incubated at 0 °C for 1 h and then processed as described in the legend to Fig. 1.

ATP for Fe compared with that of citrate to determine the free Fe]. Rigorous analysis requires that there be equilibrium between the free Fe and the mitochondrial Fe³⁺ receptor; although this is almost certainly not achieved in the 1 h incubation, the error will underestimate the association constant.

We also investigated the binding of Fe²⁺ to mitochondria: using ascorbate or dithionite to reduce the iron, we found only non-specific binding (Fig. 2).

Since the mitochondrial receptor binds Fe which is free of the ligand to which it was initially complexed, one would anticipate that Fe binding by the mitochondria would be directly proportional to the free Fe in equilibrium with the ligand-Fe complex (and inversely proportional to the affinity of the donating ligand for Fe). Although the strongest ligands studied (i.e. PP_i and nitrilotriacetate) donated less Fe to mitochondria than weaker ligands [e.g. GTP and ATP, which nevertheless are strong enough ligands to give a clear plateau in saturation experiments (Fig. 2); their binding thus containing a negligible non-specific component], there was not a close inverse correlation between affinity of in-

Fig. 3. Scatchard plot analysis of binding of Fe to mitochondria

Rat liver mitochondria, as described in the legend to Fig. 1, were incubated at 0 °C for 1 h with 60 μM -Fe complexed to citrate in buffer A at pH 7.4; the molar ratio of citrate/Fe ranged from 4:1 to 64:1. After the incubation, samples were processed as described in the legend to Fig. 1.

dividual ligands for Fe and the amount of Fe which they yielded to mitochondria (Table 2).

DISCUSSION

Fe, on first entering the cell, mixes transiently in a chelatable pool in which it is bound to low- M_r ligands (Pippard *et al.*, 1982; Mulligan *et al.*, 1986). This appears to be the case in reticulocytes, lymphocytes and Chang cells and also in cells derived from bone marrow, liver, kidney, brain and muscle (Primovich & Thomas, 1968; Mansour *et al.*, 1972; Borova *et al.*, 1973; White *et al.*, 1976; Nunez *et al.*, 1978; Milsom & Batey, 1979; LaCross & Linder, 1980; Pollack *et al.*, 1985; Mulligan *et al.*, 1986; Bomford *et al.*, 1986). The low- M_r Fe pool in lymphocytes has been quantified by Bomford *et al.* (1986), who found the total Fe in the cytosol to be 15% greater than that in the combined high- M_r Fe-containing pools of that cell. It has also been quantified by Mulligan *et al.* (1986) who found variable amounts in different tissues with as little as 8% of the Fe in the liver and as much as 36% of that in brain chelatable and associated with low- M_r ligands.

The ligands for Fe in this low- M_r chelatable pool have not been unequivocally elucidated. The earliest suggestion was that ATP is the principal ligand (Bartlett, 1970); other suggestions have included GTP, PP_i , amino acids,

Table 2. Comparison of the relative affinity of designated ligands for Fe and the amount of Fe provided to mitochondria

R is the affinity of the designated ligand for Fe compared with the affinity of acetohydroxamate for Fe, with the acetohydroxamate affinity given an arbitrary value of 1. The calculation is described in the Materials and methods section. Fe binding is measured after incubation of mitochondria with the designated ligands (4:1 molar ratio of ligand/Fe; 60 μM -Fe, in buffer A at pH 7.4). After the incubation, mitochondria were processed as described in the legend to Fig. 1. Fe bound is the average of two independent determinations, except for ATP (four determinations) and PP_i (five determinations). NTA, nitrilotriacetate.

Ligand	R	Fe bound ($\mu\text{mol/g}$ of protein)
DPG	0.0048	0.75
ADP	0.0085	0.75
GTP	0.015	0.65
ATP	0.024	0.83
Citrate	0.32	0.16
NTA	0.69	0.018
PP_i	4.7	0.056

polypeptides, proteins and growth factors (Konopka & Szotor, 1972; Boulard *et al.*, 1972; Bartlett, 1976; Fernandez-Pol, 1978; Pickart & Thaler, 1980; Baakern *et al.*, 1985; Nilsen & Romslo, 1985; Jones *et al.*, 1986; Inoue *et al.*, 1987).

Recent observations have again suggested that ATP is the key constituent of that pool. (1) ATP can remove Fe from transferrin at pH 7, at molar ratios of ATP/transferrin which could readily be achieved in the cell (Pollack & Weaver, 1986). (2) ATP is the principal ligand for low- M_r iron in the reticulocyte cytosol (Weaver & Pollack, 1989). This latter determination was made with a procedure which would readily have detected the Fe complexes of 2,3-DPG and GTP as well as those of citrate and PP_i (J. Weaver, H. Zhan & S. Pollack, unpublished work), none of which were found.

Because the ATP-Fe complex originates in a fraction of reticulocyte cytosol which with a pulse of ^{59}Fe gains Fe quickly and with a chase loses it rapidly, it has been suggested that ATP-Fe is part of a transit pool (Pollack & Campana, 1981). The interaction of the Fe complex of ATP with mitochondria was investigated in the present experiments in an effort to gain further insight into the possible role of ATP in intracellular Fe transport. Fe^{3+} bound to specific mitochondrial receptors; the Fe bound free of the ATP. These receptors also accepted Fe^{3+} bound to other ligands. Although there was an approximate inverse correlation between the amount of Fe bound specifically by the mitochondria and the order of magnitude of the affinity for Fe of the presenting ligand (thus, a direct correlation between Fe bound by the mitochondria and the free Fe in equilibrium with the ligand-Fe complex), the correlation was poor when ligands with affinities differing by less than one order of magnitude were compared. This suggests that an interaction between the ligand-Fe complex and the mitochondrial membrane may precede binding of Fe to the receptor and that this interaction may control the amount of Fe bound after short incubation times, rather than the

available free Fe (though with limits dictated by the order of magnitude of the free Fe concentration).

ATP-Fe was the most efficient iron donor to the specific binding sites for Fe^{3+} on mitochondria when compared with other ligands in its class. The amount of Fe provided to mitochondria when a ligand must first obtain it from transferrin (Konopka, 1978; Konopka & Romslo, 1980, 1981; Nilsen & Romslo, 1984*a,b*, 1985) is not readily comparable since it will be determined by at least three partially independent variables: the efficiency with which the ligand mobilizes Fe from transferrin; the intrinsic affinity of the ligand for Fe; and an interaction between the ligand and the mitochondria, probably involving an interaction of the ligand-Fe complex with the mitochondria before release of the Fe from the complex to the Fe^{3+} receptor of the mitochondria.

In contrast with Fe^{3+} , Fe^{2+} was not specifically bound by mitochondria. This suggests that, *in vivo*, Fe^{2+} may not be an important donor of Fe for haem synthesis. Although some experiments have suggested that low- M_r cytosolic Fe may exist primarily as Fe^{2+} (Egyed & Saltman, 1984; Eguchi & Saltman, 1984; Zhan *et al.*, 1989), all of the observations concerning cytosolic Fe^{2+} have been made in the presence of an agent which sequesters Fe^{2+} . Since a sequestering agent can shift the equilibrium between Fe^{2+} and Fe^{3+} , the amount of Fe^{2+} present in the cytosol in the absence of the sequestering agent is a matter for conjecture. Fe^{2+} exchange rates are much more rapid than those of Fe^{3+} ; thus a pool in which Fe^{3+} is dominant may serve to allow a more controlled processing of Fe and a more limited generation of free radicals.

One proposal concerning mitochondria in eukaryotic cells is that they originated as intracellular bacteria in prokaryotic cells. An intriguing analogy may be discerned, with ATP analogous to a bacterial siderophore and the Fe^{3+} receptor to the bacterial surface receptor for the siderophore-Fe complex. We have found that mitochondria bind Fe without the ATP (the siderophore). In some instances, Fe is taken up by bacteria without the siderophore binding to the bacterial surface (Hussein *et al.*, 1981) and in some instances without a siderophore altogether (Russell & Holmes, 1983). There is no published information concerning bacterial receptors for Fe^{3+} (Neilands, 1981). The present work makes it seem probable that such will be found.

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