Low- M_r iron isolated from guinea pig reticulocytes as AMP–Fe and ATP–Fe complexes

Janet WEAVER and Simeon POLLACK*

Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

Guinea pig reticulocytes were pulse-labelled with ^{59}Fe bound to transferrin. Haemolysates prepared from these reticulocytes were subjected to rapid $(\text{NH}_4)_2\text{SO}_4$ precipitation and then chromatography on an anion-exchange resin. ATP-bound ^{59}Fe was the dominant species in the reticulocyte cytosol; 2,3-bisphosphoglycerate and GTP iron complexes were not detected despite the fact that these were stable with $(\text{NH}_4)_2\text{SO}_4$ precipitation and readily detected with anion-exchange chromatography. AMP-bound Fe was a minor component of the cytosol following rapid $(\text{NH}_4)_2\text{SO}_4$ precipitation, and the major component when iron was released from transferrin by haemolysates. We speculate that ATP–Fe may be degraded in the cell to permit utilization of its iron for haem synthesis.

INTRODUCTION

Iron first entering the cell mixes in a ‘chelatable’ pool before it is incorporated into haem and ferritin (Pippard *et al.*, 1982). Mulligan *et al.* (1986) quantified the iron in this pool by measuring the iron extracted by desferrioxamine from tissue homogenates. They found that the amount of chelatable iron correlated with the amount which was ultrafilterable and thus showed it to be bound to low- M_r ligands. Other investigators have quantified the low- M_r iron pool in mammalian cells using molecular sieve chromatography and ultrafiltration, and by subtracting from total iron the sum of the iron in high- M_r pools (thus identifying that in a residual, presumably low- M_r , pool) (Primosigh & Thomas, 1968; Mansour *et al.*, 1972; Borova *et al.*, 1973; Nunez *et al.*, 1978; Milsom & Batey, 1979; LaCross & Linder, 1980; Bomford *et al.*, 1986; Mulligan *et al.*, 1986).

Despite these efforts, the ligands which constitute the low- M_r iron pool have not been identified (and perhaps because of this the significance of the pool has been questioned) (Crichton, 1984; Bremond *et al.*, 1988). Some investigators have suggested that the low- M_r pool of iron in the cell is the sum of all the potential low- M_r iron ligands in the cell (Jacobs, 1977); others that the pool may be impossible to characterize because of the labile nature of the equilibria involved (May *et al.*, 1978) and that the problem may be best approached by computer modelling. Other investigators have detected or postulated specific compounds as major constituents of the low- M_r iron pool. ATP- and GTP-bound iron have been suggested to be major constituents of the low- M_r iron pool (Bartlett, 1970, 1976; Konopka & Szotov, 1972). However, the experiments supporting that suggestion included acid extraction of haemoglobin containing solutions, a procedure which was shown (Meyers *et al.*, 1973) to liberate iron from haem with that iron then binding to nucleotides. Nilsen & Romslo (1985) have postulated that pyrophosphate serves as the intermediary transport ligand for intracellular iron. But there

is a vanishingly small concentration of pyrophosphate in the cell and no evidence that sequestered sites with higher concentrations exist. Other compounds which have been identified as ligands for iron include amino acids, polypeptides, proteins and uncharacterized growth factors (Boulard *et al.*, 1972; Fernandez-Pol, 1978; Pickart & Thaler, 1980; Bakkeren *et al.*, 1985; Jones *et al.*, 1986; Inoue *et al.*, 1987). But their roles, if any, as ligands for intracellular iron transport are not known.

Earlier work from our laboratory (Pollack *et al.*, 1985) suggested that AMP–Fe was the major constituent of the low- M_r pool. Reticulocytes, incubated with ^{59}Fe -labelled transferrin, then haemolysed and subjected to molecular sieve chromatography, yielded a low- M_r ^{59}Fe peak; pulse-chase experiments showed that peak to have a rapid turnover of its ^{59}Fe . When purified by ion-exchange and paper chromatography, the ^{59}Fe in that peak co-migrated with AMP.

Nevertheless, it seemed implausible that AMP would dominate a low- M_r iron pool; ATP binds iron more strongly and is present in high concentration in cell cytosol. Also, reticulocyte haemolysates release iron from transferrin at pH 7, an activity that derives from the interaction of the ATP with an $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction of the haemolysate (Pollack & Weaver, 1986); an equivalent concentration of AMP interacting with the $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction is virtually inert.

In the present work we first identified the dominant ligand which binds iron when it has been released from transferrin by haemolysates *in vitro*; it proved to be AMP and to persist as an AMP–Fe complex even when an excess of ATP had been added. The binding strength for iron of the added ATP is such that it should have removed the iron from AMP. Since it did not, the possibility that phosphatases in the haemolysate might be generating AMP–Fe from ATP–Fe was considered. The low- M_r ^{59}Fe complex in intact reticulocytes was studied using rapid $(\text{NH}_4)_2\text{SO}_4$ precipitation to separate potential phosphatases from low- M_r iron ligands. With this procedure ATP proved to be the dominant ligand for

Abbreviation used: 2,3-DPG, 2,3-bisphospho-D-glyceric acid.

* To whom correspondence and reprint requests should be addressed.

iron in reticulocyte cytosol, but AMP-Fe was still present. We suggest that ATP interacts with transferrin to effect the initial release of iron and that it continues as the principal ligand for iron in the cytosol. Whether degradation of the ATP-Fe complex to an AMP-Fe species occurs *in vivo* and makes the iron more available for haem synthesis or whether that degradation is an artifact of the procedure *in vitro* remains to be determined.

EXPERIMENTAL

Materials

Guinea pig (Hartley, male) blood was collected in 0.1 M-NaCl/0.02 M-Hepes (pH 7.0) (buffer 1) containing heparin, washed three times with buffer 1, filtered through 120-mesh nylon cloth with the first wash, and through 20-mesh nylon cloth with the second and third washes to remove the visible buffy coat.

Reticulocyte-rich blood was obtained by repeated bleeding (unless otherwise specified) to achieve reticulocyte counts of 28%–40%. Human apotransferrin (Calbiochem-Behring) was loaded with ^{59}Fe as previously described (Pollack & Weaver, 1985). ATP, GTP, ADP, AMP, 2,3-DPG and Hepes were obtained from Sigma.

Incubation and ultrafiltration of haemolysate

Washed cells were haemolysed by freeze-thaw (liquid N_2) in 1 vol. of buffer 1 and centrifuged at 36000 *g* for 30 min at 4 °C. Immediately after preparation, 1 vol. of haemolysate was mixed with 3 vol. of buffer 1 containing bovine serum albumin (Sigma) at 80 mg/ml. Then 0.2 ml of 0.05 mM-transferrin saturated with ^{59}Fe was added for each 1 ml of haemolysate, and the mixture was incubated at 37 °C for 1 h. The incubation mixture was transferred to Amicon CF-25 filter cones, which had been soaked in deionized water, and centrifuged at 211 *g* at 4 °C for 1 h. Blanks contained additional buffer in place of haemolysate.

DEAE-Sephadex chromatography

DEAE-Sephadex (7 g) was packed into a 1.6 cm diameter column and equilibrated with 0.05 M-NaCl/0.01 M-Hepes, pH 8.0. The ultrafiltrate, immediately after preparation, was loaded on the column and eluted, at 6 °C, with a linear gradient of 0.05 M–0.5 M-NaCl in 0.01 M-Hepes, pH 8.0 (gassed with N_2), at a rate of 12 ml/h; 16 min fractions were collected, counted in a γ -radiation counter, and absorbance at 260 nm was determined. The major ^{59}Fe peak was lyophilized overnight. The ultrafiltrate of the blank was simultaneously eluted from an identical DEAE-Sephadex column, and equivalent fractions were collected and lyophilized.

Paper chromatography of DEAE-Sephadex peak

Whatman 3 MM paper was washed by development in methanol/formic acid/water (7:2:1, by vol.) (solvent 1) followed by deionized water, solvent 1, and deionized water. Lyophilized samples and blanks were redissolved in deionized water to about 10% of original volume, and applied under N_2 . To remove excess salt, after the sample had been applied, plates or paper were dipped in methanol and allowed to dry before development. For analytical work, 2 μl samples were spotted on paper. After development, chromatograms were cut into 0.8 cm

wide strips and ^{59}Fe radioactivity was counted. For preparative work, sample was applied on the paper as a streak at the origin. After development in solvent 1, the paper was scanned under u.v. The u.v.-absorbing area of the paper was cut out and eluted by allowing solvent 1 to drip through it overnight. The corresponding area of the blank was cut out and eluted.

Analysis of samples

The solvent 1 eluate was lyophilized overnight, redissolved in 2–4 ml of deionized water, and filtered. The absorbance spectrum was determined in a Cary model 17D spectrophotometer. Samples were assayed for pentose using the orcinol reaction (Dische, 1955), and for phosphate (Leloir & Cardini, 1957). 5'-AMP was used as a standard, and the blanks were those which had been carried through the entire procedure.

Hydrolysis of the molecule was carried out by incubation in 1 M-HCl for 1 h at 100 °C (Parrish, 1972), and the products analysed by ascending paper chromatography on Whatman 3MM paper. Solvent systems used were: A, isobutyric acid/0.5 M- NH_3 (5:3, v/v) (Wyatt, 1955); B, methanol/ethanol/conc. HCl/water (50:25:6:19, by vol.) (Parrish, 1972); C, ethyl acetate/propan-1-ol/water (4:1:2, by vol.) (Hall, 1963).

Preparation of samples for $(\text{NH}_4)_2\text{SO}_4$ precipitation

Washed red blood cells, suspended in 1.5 vol. of buffer 1, were lysed by three cycles of freeze-thaw, centrifuged (35600 *g* for 30 min), and the supernatant was stored in liquid N_2 until use. Then, 17 ml of the haemolysate, or 8.5 ml of the haemolysate plus 8.5 ml of 2 mM-ATP, was incubated with 1.7 ml of ^{59}Fe -transferrin (0.05 mM) at 37 °C for 10 min in a shaking water bath.

For studies of the iron released from transferrin by the intact reticulocyte, washed cells containing about 40% reticulocytes, suspended in an equal volume of buffer 1, were incubated as above with ^{59}Fe -transferrin. Cells were then washed three times with ice-cold buffer 1, lysed by freeze-thaw and centrifuged at 35600 *g* for 30 min.

$(\text{NH}_4)_2\text{SO}_4$ precipitation and ion-exchange chromatography

Dry $(\text{NH}_4)_2\text{SO}_4$ was added to saturation (0.71 g/ml) to ice-cold haemolysates or standards. The pH was monitored during this addition and found to rise to approx. 8. The mixture was centrifuged (35600 *g* for 30 min) and the supernatant diluted to conductivity of less than 5 mS (approx. 300-fold dilution). The dilute supernatant was applied to a 1 cm $\times$ 25 cm column of Bio-Rad AG 1-X8 resin which had been converted to the formate form by perfusion with 6 M-formate (6 M-formic acid/6 M-ammonium formate; 4:1, v/v) followed by glass-distilled water until the conductivity of the effluent was less than 5 mS. After application of the sample, the column was washed with water and eluted with a gradient of 0–4 M- or 0–6 M-formate (500 ml each), at a rate of 1 ml/min. The 0–4 M gradient was used with preparations containing ATP-Fe or ATP with transferrin, alone or with unlabelled haemolysate. For experiments in which Fe was taken up from transferrin by intact reticulocytes and for all standards, the 0–6 M gradient was used. Each fraction was analysed for ^{59}Fe (Tracor γ -radiation counter) and 260 nm absorbance (Beckman DU-2 spectrophotometer).

For experiments leading to iron determinations, ATP was pretreated with Chelex (Sigma) and solutions were pretreated with dithizone to remove iron. Glass-distilled water was used throughout.

Preparation of standards

To prepare complexes of ^{59}Fe with nucleotides or 2,3-DPG, these were dissolved in ice-cold 0.1 M-NaCl/0.1 M-Hepes (pH 7.0) and $^{59}\text{FeCl}_3$ in 1 M-HCl was slowly added, on ice, with constant stirring and addition of 1 M-NaOH to maintain the pH. The molar ratio of ligand to iron was 10:1, to prevent the formation of polynuclear iron (Mansour *et al.*, 1985).

^{59}Fe -haemoglobin was prepared by incubating reticulocytes with ^{59}Fe -transferrin at 37 °C for 1 h. The haemoglobin was purified from the freeze-thaw haemolysate by gel exclusion chromatography on Ultrogel AcA 44 (LKB) in buffer 1.

RESULTS

When ^{59}Fe -labelled transferrin was incubated with haemolysate at 37 °C and then subjected to ultrafiltration, an average of 4.9% of the iron was released (10 experiments). (^{59}Fe recovered in ultrafiltrates in the absence of haemolysate was less than 0.1%.) The ^{59}Fe -containing ultrafiltrate was applied to DEAE-Sephadex and eluted with a salt gradient, 0.05–0.5 M-NaCl in 0.01 M-Hepes, pH 8. ^{59}Fe was eluted from the column in a single major peak coincident with a 260 nm absorbing peak, at a buffer conductivity of 18–20 mS (Fig. 1). Recovery of ^{59}Fe applied to the column averaged 41% (10 experiments). A small ^{59}Fe peak which was eluted prior to the major peak, without coincident 260 nm absorbing material, was not studied further. A large double peak of 260 nm absorbance following the major ^{59}Fe peak appeared to be ATP, based on elution position and ribose:phosphate ratio. ^{59}Fe applied to the column as ferric chloride was not eluted in the salt gradient.

The DEAE peak was concentrated by lyophilization and further purified by paper chromatography, first in solvent 1, where the radioactivity migrated, together with the u.v.-absorbing material, with an R_F of 0.37 (the ^{59}Fe in a 10:1 AMP-Fe complex made *in vitro* had an R_F of 0.41–0.45), then in 1 M-LiCl where the R_F was 0.87 (the ^{59}Fe in a 10:1 AMP-Fe complex stayed at the origin; this is probably due to formation of polynuclear iron in the prepared complex).

The material was eluted by solvent 1. Two of the eluates were hydrolysed in acid and then subjected to paper chromatography; a single u.v.-absorbing spot moved with an R_F identical to adenine in three solvents. After development in solvent B and elution in 0.1 M-HCl, the spectrum of the compound at pH 1, 7 and 13 was determined, and the shifts observed were identical to those of adenine. Five of the solvent 1 eluates were analysed for pentose and phosphate, and the adenine concentration was determined by 255 nm absorbance. These proved to be in a 1:1:1 molar ratio.

The possibility that the AMP-Fe complex obtained was the result of an idiosyncratic property of the purification scheme employed was considered. Therefore, an alternative purification scheme was tried. Haemolysates were incubated with ^{59}Fe -labelled transferrin and the mixture precipitated with $(\text{NH}_4)_2\text{SO}_4$. An average of 6.9% of the counts were not precipitated

Fig. 1. Elution from DEAE-Sephadex of ^{59}Fe -labelled guinea pig haemolysate

The ultrafiltrate obtained following incubation of haemolysate and ^{59}Fe -labelled transferrin was applied to DEAE-Sephadex and eluted, at 6 °C, with a linear gradient of 0.05–0.50 M-NaCl in 0.01 M-Hepes, pH 8.0. O—O, A_{260} ; Δ—Δ, radioactivity.

Fig. 2. Elution from Bio-Rad AG 1-X8 of the $(\text{NH}_4)_2\text{SO}_4$ supernatant of ^{59}Fe -labelled guinea pig haemolysate

Guinea pig haemolysate (17 ml) was incubated with 1.7 ml of ^{59}Fe -transferrin (639 000 c.p.m.), subjected to precipitation with $(\text{NH}_4)_2\text{SO}_4$ and the supernatant (containing 5.6% of the radioactivity) chromatographed on Bio-Rad AG 1-X8, formate form, in a gradient of 0–4 M-formate. Of the radioactivity applied, 42% was recovered from the column, 20% in the first peak. Conductivity of peaks was 9 mS and 46 mS. —, A_{260} ; ----, radioactivity.

with $(\text{NH}_4)_2\text{SO}_4$ (six experiments). The $(\text{NH}_4)_2\text{SO}_4$ supernatant was applied to an AG 1-X8 column and eluted with an ammonium formate gradient. The major peak of radioactivity eluted at a buffer conductivity of 9 mS and a minor peak at 46 mS (Fig. 2). Iron complexes of ATP, ADP, AMP, GTP and 2,3-DPG applied to this column were eluted at distinct positions (Fig. 3), and the major peak eluted when haemolysate was applied to the column coincided with the AMP-Fe. However, a very small peak was present at 46 mS, the elution position of ATP-Fe. ATP was added to the haemolysate and the mixture incubated with transferrin, then precipitated with $(\text{NH}_4)_2\text{SO}_4$ and then applied to the column and eluted (Fig. 4). A distinct ATP-Fe peak was seen, but the AMP-Fe peak continued to be present. When AMP- ^{59}Fe (10:1 complex) was incubated with a 10-fold molar excess of ATP for 1 h at 37 °C there was complete transfer of the ^{59}Fe to the ATP. ATP added to transferrin

Fig. 3. Composite graph of the elution patterns of several low- M_r iron complexes from Bio-Rad AG 1-X8 formate

10:1 complexes were precipitated with $(\text{NH}_4)_2\text{SO}_4$ and the supernatant, suitably diluted, was applied to a 1 cm $\times$ 25 cm column and eluted with a gradient of 0–6 M-formate (500 ml each) at 64 ml/h. The graph is normalized to show % recovery in each fraction of the ^{59}Fe applied to the column. Overall recovery of radioactivity for each complex was: AMP-Fe, 38%; ADP-Fe, 49%; ATP-Fe, 82%; GTP-Fe, 79%; 2,3-DPG-Fe, 70%; FeCl_3 , 33%. $\bullet$, ATP-Fe; $\circ$, ADP-Fe; $\square$, AMP-Fe; $\blacksquare$, GTP-Fe; $\triangle$, 2,3-DPG-Fe; $\blacktriangle$, FeCl_3 . Conductivity: AMP-Fe, 9 mS; ADP-Fe, 19 mS; ATP-Fe, 46 mS; GTP-Fe, 63.5 mS; 2,3-DPG-Fe, 16.5 mS.

Fig. 4. Elution from Bio-Rad AG 1-X8 of the $(\text{NH}_4)_2\text{SO}_4$ supernatant of ATP incubated with ^{59}Fe -transferrin and guinea pig haemolysate

Guinea pig haemolysate (8.5 ml) mixed with 8.5 ml of 2 mM-ATP was incubated with 1.7 ml of ^{59}Fe -transferrin (2673000 c.p.m.) and treated as in Fig. 3. The $(\text{NH}_4)_2\text{SO}_4$ supernatant contained 23% of the radioactivity; 66% of the radioactivity applied was recovered from the column, 20% in the first peak. Conductivity of peaks was 10 mS and 48 mS. —, A_{260} ; ---, radioactivity.

in the absence of haemolysate yielded only an ATP-Fe complex (Fig. 5).

The AMP-Fe complex obtained both in earlier experiments (when reticulocytes had been incubated with ^{59}Fe -transferrin and that haemolysate subjected to molecular sieve and ion-exchange chromatography) and in these experiments could be interpreted as showing that

Fig. 5. Elution from Bio-Rad AG 1-X8 of the $(\text{NH}_4)_2\text{SO}_4$ supernatant of ATP incubated with ^{59}Fe -transferrin

A solution (17 ml) of 1 mM-ATP in 0.1 M-NaCl/0.02 M-Hepes, pH 7, was incubated with 1.7 ml of ^{59}Fe -transferrin (1399000 c.p.m.) and treated as in Fig. 3. The $(\text{NH}_4)_2\text{SO}_4$ supernatant contained 80% of the radioactivity; 89% of the radioactivity applied was recovered from the column. Analysis of the four peaks for pentose, phosphate and iron showed them to be, in order of elution, AMP, ADP, ATP and ATP-Fe (1:1). Conductivities were, respectively, 5 mS, 17 mS, 48 mS and 49 mS. —, A_{260} ; ---, radioactivity.

ATP-Fe, in the presence of the phosphatases of the intact red cell or the haemolysate, is rapidly hydrolysed to generate a less phosphorylated iron-nucleotide com-

Fig. 6. Elution pattern of $(\text{NH}_4)_2\text{SO}_4$ supernatant of guinea pig reticulocyte haemolysate labelled with ^{59}Fe

Conditions: Bio-Rad AG 1-X8 formate column (1 cm $\times$ 25 cm), 0–6 M-formate gradient, 500 ml each, flow rate 64 ml/h. Conductivity of peaks was 7.5 mS and 48 mS.

Fig. 7. Comparison of the distribution of ^{59}Fe in column fractions in experiments which resulted in low and high overall recovery of ^{59}Fe

Because gradients and fraction sizes differed, % of iron recovered is plotted as a function of buffer conductivity. (a) $(\text{NH}_4)_2\text{SO}_4$ supernatant of ^{59}Fe -labelled guinea pig haemolysate, from cells which contained 40% reticulocytes, chromatographed on a 1 cm $\times$ 25 cm column of Bio-Rad AG 1-X8 formate, in 0–4 M-formate, 500 ml each, 1.07 ml/min. Of the iron applied to the column 29% was recovered, 18% in the AMP peak. (b) A similar preparation from cells which contained 37% reticulocytes, on an identical column, eluted with 0–6 M-formate; 77% of the iron was recovered, 10% in the AMP peak.

plex. We, therefore, restudied the low- M_r iron complex of the whole reticulocyte using $(\text{NH}_4)_2\text{SO}_4$ precipitation in an effort to rapidly separate protein phosphatases from nucleotides.

Reticulocyte-rich guinea pig red blood cells were incubated with ^{59}Fe -transferrin, haemolysed by freeze-thaw, and proteins precipitated by $(\text{NH}_4)_2\text{SO}_4$. After centrifugation, 10–23% (mean of 13.1% in four experiments) of the iron taken up by the cells remained in the $(\text{NH}_4)_2\text{SO}_4$ supernatant. When the supernatant was chromatographed, the same two iron peaks appeared

Fig. 8. Elution from Bio-Rad AG 1-X8 of the $(\text{NH}_4)_2\text{SO}_4$ supernatant of guinea pig reticulocytes incubated with ATP- ^{59}Fe

ATP- ^{59}Fe (17 μmol of ATP; 10:1 complex) was mixed with 5 ml of guinea pig red cells (reticulocyte count 39%, following the administration of phenylhydrazine) and 7.5 ml of buffer 1. The mixture was warmed in a 37 $^\circ\text{C}$ bath for 1 min (reaching a temperature of 21 $^\circ\text{C}$) and then subjected to freeze-thaw haemolysis and $(\text{NH}_4)_2\text{SO}_4$ precipitation. The supernatant was applied to a Bio-Rad AG 1-X8 column and eluted with a 0–6 M-formate gradient (500 ml each); 4.7 ml fractions were collected. Of the ^{59}Fe applied to the column 46.9% was recovered. —, A_{260} ; ----, radioactivity.

as in the earlier haemolysate experiments, with ATP-Fe the dominant species (twice as much ATP-Fe as AMP-Fe; average of three experiments) (Fig. 6). Recovery of ^{59}Fe from the columns varied from 29% to 70% in three experiments, with higher recoveries observed when a larger proportion of the iron was in the ATP-Fe peak (Fig. 7). This suggested that the reticulocyte's phosphatases hydrolyse ATP-Fe to generate AMP-Fe and also that the hydrolysis diminishes the recoverability of low- M_r iron from the cytosol. In order to examine the possibility further, ATP- ^{59}Fe was added to reticulocytes; the mixture was then subjected to freeze-thaw haemolysis and $(\text{NH}_4)_2\text{SO}_4$ precipitation, and the supernatant applied to an AG 1-X8 column and eluted. ATP-Fe and AMP-Fe were eluted from the column (Fig. 8). Recovery of ^{59}Fe applied to the column was 49.6% and 61.7% in two experiments; when ATP- ^{59}Fe was applied to the AG 1-X8 column recovery of ^{59}Fe was 92.8% and 90.6% in two experiments and no AMP-Fe was present.

As expected, there was no significant release of iron from haemoglobin with the $(\text{NH}_4)_2\text{SO}_4$ precipitation; ^{59}Fe -labelled haemoglobin from guinea pig reticulocytes, containing the amount of radioactivity usually contained in total haemolysate in these experiments, and mixed with unlabelled haemolysate, was precipitated with $(\text{NH}_4)_2\text{SO}_4$. Only 1.1% of the ^{59}Fe remained in the supernatant (10–23% was in the supernatant of haemolysates).

DISCUSSION

In the present work we find that the major identifiable ligand which binds iron in the reticulocyte cytosol is ATP, but that substantial amounts of an AMP-Fe complex are also present under all conditions studied. The existence of the ATP-Fe complex is consistent with the many observations that have shown ATP to be effective at removing iron from transferrin (Pollack *et al.*,

1977; Morgan, 1979; Carver & Frieden, 1978). It seems plausible that it would continue to bind iron after its release from transferrin. The AMP-Fe complex is more difficult to rationalize. It could be the consequence of phosphatases, active *in vitro* when the cell is broken, but not in the intact cell. However, several arguments can be made for the physiological significance of the AMP-Fe complex. ATP binds iron at pH 7 with a formation constant of 10^{29} (Romslo, 1980). Such avidity might interdict the transfer of the iron from ATP to any other ligand; thus destruction of the ATP could be required for the iron to be released. Also, the more highly charged and bulkier ATP-Fe complex may be less accessible to apoferritin and to mitochondria.

Our failure to observe GTP-Fe and DPG-Fe is noteworthy. Both GTP and DPG form robust iron complexes which are identified easily following $(\text{NH}_4)_2\text{SO}_4$ and AGI-X8 chromatography. Moreover, DPG is almost as active as ATP in releasing iron from transferrin. Also GTP binds iron more effectively than ATP when the iron is added to a mixture of the two (Bartlett, 1976). Compartmentalization of GTP and DPG is a possible explanation for their apparent failure to function as significant iron ligands in the cell. The bulk of DPG in the cell is complexed to haemoglobin (although this varies with oxygenation) (Bunn *et al.*, 1971). No single major binding site for GTP exists, but the collective effect of numerous minor binding sites may make its free concentration small.

The unrecovered iron, which in the intact reticulocyte experiments ranged from 20 to 70 %, may be the result of transfer of iron from ATP to AMP, and its subsequent transfer from the more weakly binding AMP to other sites, perhaps including glass and column resins. But it is also possible that other significant low- M_r cytosolic iron ligands remain to be identified.

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