Chelation of transferrin iron by desferrioxamine in K562 cells

The partition of iron between ferrioxamine and ferritin

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In this study we have determined whether desferrioxamine can chelate iron delivered to human leukaemic cells by the transferrin endocytic cycle. The cellular uptake of desferrioxamine was investigated by an indirect method in which the conversion of repeated pulses of [^{59}Fe]transferrin to [^{59}Fe]ferrioxamine was determined at two concentrations of the drug. Maximum generation of [^{59}Fe]ferrioxamine occurred in cells exposed to either 100 μM - or 500 μM -desferrioxamine after 40–60 min. Thereafter (up to 180 min) [^{59}Fe]ferrioxamine levels remained steady with 20 % of a ^{59}Fe pulse partitioning to chelator at 100 μM and 50 % at 500 μM . Of the cellular [^{59}Fe]ferrioxamine loss 50 % occurred within 90–120 min. In cells preloaded with desferrioxamine for 1 or 4 h the partitioning of iron during a 3 h incubation with [^{59}Fe]transferrin was dependent upon the extracellular concentration of the chelator. Above 1 mM more than 80 % of entering iron was converted to ferrioxamine and less than 5 % partitioned to ferritin. Below this concentration (50–500 μM) a proportion of the iron became ferritin associated (7–41 %). There was a linear increase in the total amount of intracellular [^{59}Fe]ferrioxamine in accordance with cellular iron uptake showing that transferrin continued to cycle in the presence of high concentrations of desferrioxamine. The uptake of iron and generation of ferrioxamine were markedly reduced by 5 mM-methylamine, which prevented endosome acidification and uncoupling of iron from endocytosed transferrin.

INTRODUCTION

The physiological entry of iron into cells is dependent upon the presence of specific cell surface receptors for the plasma iron-transport protein transferrin (Jandl & Katz, 1963). Following binding, the receptor–ligand complex is endocytosed (Morgan & Appleton, 1969; Karin & Mintz, 1981) and the iron released from the two iron-binding sites of the protein (Ciechanover *et al.*, 1983; Bomford *et al.*, 1985). This is achieved through a process of reduction and/or protonation in the intravesicular acid environment (van Renswoude *et al.*, 1982) thought to be generated by an ATP–Mg $^{2+}$ -dependent mechanism (Yamashiro *et al.*, 1983). The point in the cycle at which iron is released from the endosome is unknown, but in view of the toxic nature of free iron (Halliwell & Gutteridge, 1984) it is unlikely that it remains, at any time, in an uncomplexed form. Implicit in the proposed mechanisms of iron release from transferrin is the need for an acceptor molecule (Aisen & Listowsky, 1980). Presumably such species are required both within the endosome and across its membrane barrier in the cytosol.

The concept of a cytosolic chelatable iron pool into which transferrin iron is fed *en route* to functional haem-containing enzymes or the storage compound ferritin has been inferred (Jacobs, 1977) from experiments in which low-molecular-mass iron has been identified in a number of cell types (Lipschitz *et al.*, 1971; White *et al.*, 1976a; Octave *et al.*, 1983). These studies have used the permeant iron-chelating agent desferrioxamine in an attempt to define the intracellular sources of iron available for chelation. However, the specific relationship between chelatable iron and the dynamics of the transferrin cycle have not been investigated.

In the present study we have examined the kinetics of desferrioxamine uptake into K562 cells by determining the extracellular concentration of chelator and duration of exposure required to achieve maximum binding of iron entering the cell on transferrin. In additional experiments the compound was used as a competitive acceptor molecule for iron liberated during the transferrin cycle to characterize the kinetics of iron release from the endosome and the partition of iron between transferrin, chelatable and ferritin pools. The inclusion of a lysosomotropic agent which prevents acidification of the endosome and iron release from transferrin has allowed us to determine whether desferrioxamine has direct access to iron while it remains coupled to the protein.

EXPERIMENTAL PROCEDURES

Materials

$^{59}\text{FeCl}_3$ (average specific radioactivity 28 mCi/mg) was from Amersham International. Bovine serum albumin (BSA) (fraction V), methylamine, deoxycholic acid and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., U.K., and Dowex 1-X8 and benzyl alcohol were from BDH Chemicals. Desferrioxamine mesylate (Desferal) was supplied by Ciba Laboratories.

Cell culture

K562 cells were cultured in 75 cm 3 plastic flasks (Gibco-Nunc) in RPMI 1640 chemically-defined medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum (Gibco, Europe) (culture medium) in CO $_2$ /air (1:19) at 37 °C (Bomford *et al.*, 1986a). Cultures

were maintained in exponential-phase growth at densities of $(2-7) \times 10^5$ cells/ml.

Preparation of ^{59}Fe -labelled transferrin

Transferrin was prepared from pooled and iron-saturated human plasma as previously described (Bomford *et al.*, 1983) using DEAE-Sephadex chromatography to yield diferric transferrin with an A_{470}/A_{410} ratio greater than 1.3. Apotransferrin was prepared by repeated citrate dialysis (Young *et al.*, 1984) and made diferric with ^{59}Fe (Bomford *et al.*, 1983). Non-specifically bound iron was removed by chromatography on the anion-exchange resin Dowex 1-X8 (Cavill, 1971).

Uptake of desferrioxamine and partition of ^{59}Fe between desferrioxamine and ferritin in K562 cells

^{59}Fe -labelled human diferric transferrin was used as the source of iron for intracellular chelation by desferrioxamine. Cells were spun down and resuspended twice at 37 °C for 15 min in incubation medium to free receptors of endogenous bovine transferrin and then incubated at 37 °C for 1 or 4 h with various concentrations (20 μM –5 mM) of freshly prepared desferrioxamine in 10 ml of RPMI containing 1% BSA (incubation medium). They were then incubated at 37 °C for 3 h with 310 nM- ^{59}Fe -diferric transferrin in medium containing the original concentration of the chelator. Samples were taken at intervals, washed three times in phosphate-buffered saline (PBS) at 4 °C and either analysed immediately for ^{59}Fe ferrioxamine or ^{59}Fe ferritin or stored at –20 °C in lysis buffer (0.2% Triton X-100, 0.2% deoxycholic acid, 1 mM-phenylmethanesulphonyl fluoride, 25 mM-Hepes, pH 7.5). In some experiments after preloading with desferrioxamine the cells were incubated with 5 mM-methylamine for 20 min at 37 °C before incubation with ^{59}Fe transferrin. In other experiments, after the addition of desferrioxamine (100 μM and 500 μM), samples of cells were removed at intervals up to 3 h and pulsed for 10 min at 37 °C with 310 nM- ^{59}Fe diferric transferrin. The incubation was stopped by the addition of cold PBS, and the cell pellets were washed three times at 4 °C, and solubilized by repeated cycles of freezing and thawing in lysis buffer.

The release of ^{59}Fe ferrioxamine was studied by re-incubating the cells in culture medium at 37 °C and harvesting cells and media at intervals up to 10 h. Cells were dissolved in lysis buffer and insoluble debris was spun out at 12000 *g* for 15 min in a microcentrifuge. This resulted consistently in removal of 10% of the total counts. Two methods were used to analyse ferrioxamine concentrations in both the cell lysates and culture media.

Extraction of ferrioxamine with benzyl alcohol

Cell lysate and re-incubation media (made 0.2% in Triton X-100) were made 5 M in NaCl and assayed for ferrioxamine by extracting with an equal volume of benzyl alcohol (de Wael & Ploem, 1965). This extraction method was validated by making a standard ^{59}Fe ferrioxamine preparation by adding $^{59}\text{FeCl}_3$ to desferrioxamine in the ratio of 0.8:1 and removing unbound ^{59}Fe by anion-exchange chromatography as described above for transferrin. Of the ^{59}Fe ferrioxamine 98% was recovered from the organic phase when two extractions were performed with 0.5 min

vigorous mixing. Less than 1% of ^{59}Fe was removed from either ^{59}Fe transferrin or ^{59}Fe ferritin by this extraction procedure.

Detection of ferrioxamine by column chromatography

Cell lysates and re-incubation media were applied to a column of Sephadex G-50 (10 mm $\times$ 200 mm) equilibrated and eluted with 25 mM-Hepes, pH 7.4, 130 mM-NaCl and 1 ml fractions were collected and counted in a Compugamma counter (LKB). A standard ^{59}Fe ferrioxamine preparation was found to be eluted in fractions 21–33 and counts from samples corresponding to a peak within these fractions were taken to be ^{59}Fe ferrioxamine.

Immunoprecipitation of ^{59}Fe ferritin

An antiserum to purified human liver ferritin (Bomford *et al.*, 1977) was raised in rabbits using an immunization schedule previously described (Bomford *et al.*, 1981). The antiserum was specific for human liver ferritin as shown by radial immunodiffusion and immunoelectrophoresis. Ferritin was isolated from cell lysates using direct immunoprecipitation. Purified human liver ferritin (20 μg) was added to 0.5 ml of cell lysate followed by 30 μl of specific antiserum and incubated for 1 h at 37 °C. The tubes were then incubated overnight at 4 °C and the brown precipitate was recovered by centrifugation at 12000 *g*_{av} for 10 min. The pellets were washed four times in lysis buffer at 4 °C and counted in a gamma counter. The specificity of these conditions for precipitating ferritin was validated according to Bomford *et al.* (1986b).

RESULTS

Assessment of desferrioxamine uptake by K562 cells

Iron entering the cells on transferrin was found to be readily accessible to intracellular chelator since ^{59}Fe ferrioxamine was rapidly generated when cells pre-loaded with desferrioxamine for 1–4 h were incubated with ^{59}Fe transferrin (see below). To investigate the kinetics of entry of desferrioxamine, cells were incubated with chelator at 37 °C and pulsed at intervals with ^{59}Fe transferrin. The percentage of iron partitioning to ferrioxamine as assessed by both benzyl alcohol extraction and column chromatography increased over a 40–60 min period but thereafter remained constant (Fig. 1). When the extracellular concentration was 100 μM an equilibrium was achieved where approx. 20% of the 10 min pulse was converted to ferrioxamine. The steady level of ferrioxamine was raised to 50% at an extracellular concentration of 500 μM while the profiles of formation of ^{59}Fe ferrioxamine at the two concentrations were similar. These results show that the extracellular desferrioxamine concentration is important in determining the percentage of a pulse of transferrin iron that is bound by the chelator, while prolonged exposure to the drug does not lead to a greater percentage generation of ferrioxamine. There is, therefore, rapid uptake of desferrioxamine by K562 cells with equilibrium being reached by 40–60 min for both concentrations used.

Partition of intracellular ^{59}Fe to ferrioxamine

To characterize further the partitioning of transferrin iron to ferrioxamine, cells were pre-exposed to various concentrations of desferrioxamine (20 μM –5 mM) for

1 and 4 h and the formation of [^{59}Fe]ferrioxamine was assessed during a subsequent 3 h incubation with [^{59}Fe]transferrin. The percentage of the iron that became bound to desferrioxamine was wholly dependent upon the extracellular chelator concentration and not the length of the preincubation (Fig. 2). There was extensive (80–90 %) conversion of ^{59}Fe to ferrioxamine in the presence of 1–5 mM-desferrioxamine, whereas at lower concentrations there was a decreased partitioning of the iron to chelator so that at an extracellular concentration of 20 μM only 10 % of the iron entering the cell on transferrin was chelated. Binding of 75 % of entering iron occurred over a 10 min period when cells were pre-incubated with 5 mM-desferrioxamine. Since, under these conditions, desferrioxamine was in excess (Fig. 2) this period represented the time occupied by the binding of transferrin to the cells, endocytosis and release of iron to the chelator. The distribution of iron to ferrioxamine

Fig. 1. Uptake of desferrioxamine by K562 cells

Washed cells ($1 \times 10^6/\text{ml}$) were incubated at 37 °C in RPMI 1640, 1 % BSA (incubation medium) with either 100 μM - (●) or 500 μM - (○) desferrioxamine. At intervals aliquots (5×10^6 cells) were removed and 310 nM-[^{59}Fe]diferric transferrin was added for 10 min. Samples were then washed three times at 4 °C, lysed and assayed for [^{59}Fe]ferrioxamine. The experimental points indicated include the 10 min pulse.

Fig. 2. Generation of [^{59}Fe]ferrioxamine in K562 cells

Washed cells ($2 \times 10^6/\text{ml}$) were incubated with desferrioxamine (20 μM –5 mM) for 1 or 4 h at 37 °C in incubation medium before the addition of 310 nM-[^{59}Fe]diferric transferrin for a further 3 h. Samples (1×10^6 cells) were taken in duplicate at the times indicated, washed at 4 °C in PBS and analysed for [^{59}Fe]ferrioxamine by benzyl alcohol extraction or column chromatography as described in the Experimental procedures section. (a) 1 h preincubation; (b) 4 h preincubation. (▲) 20 μM ; (□) 100 μM ; (■) 500 μM ; (○) 1 mM; (●) 5 mM.

Fig. 3. Generation of [^{59}Fe]ferrioxamine in K562 cells

Conditions used were as described in the legend to Fig. 2. (a) 100 μM -desferrioxamine; (b) 1 mM-desferrioxamine. (■) Total ^{59}Fe uptake; (○) 1 h preincubation; (●) 4 h preincubation.

Fig. 4. Partitioning of ^{59}Fe to ferritin in K562 cells

Cells were treated under the same conditions as described in the legends to Figs. 2 and 3. [^{59}Fe]Ferritin was precipitated from solubilized cell extracts by a rabbit polyclonal anti-(human ferritin) antibody as described in the Experimental procedures section. □, 1 h preincubation with desferrioxamine; ▨, 4 h preincubation with desferrioxamine.

Fig. 5. Release of [^{59}Fe]ferrioxamine from K562 cells

Washed cells ($1 \times 10^6/\text{ml}$) were loaded with 1 mM-desferrioxamine for 4 h in incubation medium and then 310 nM-[^{59}Fe]diferric transferrin was added for a further 3 h. The cells were washed three times at 4 °C in PBS and re-incubated in chelator-free RPMI 1640 medium with 10% foetal calf serum at a density of 1×10^6 cells/ml. Aliquots (5 ml) were taken at intervals up to 10 h and a further sample at 24 h (results not shown), and medium and cells were assayed for [^{59}Fe]ferrioxamine by both extraction and column chromatography methods. (○) Intracellular [^{59}Fe]ferrioxamine; (●) extracellular [^{59}Fe]ferrioxamine.

reached steady-state levels by approx. 60 min irrespective of the preincubation time and thereafter the fraction generated remained constant. This indicates that an equilibrium between intra- and extra-cellular desferrioxamine was being maintained despite ferrioxamine formation and release of this species from the cell.

There was a linear increase in the total amount of

Fig. 6. Effect of methylamine on iron uptake and ferrioxamine formation in K562 cells

Washed cells ($1 \times 10^6/\text{ml}$) were preloaded with 1 mM-desferrioxamine for 4 h as described. Half the cells were incubated with 5 mM-methylamine for 15 min at 37 °C. Control cells were also incubated with and without methylamine in a similar manner. [^{59}Fe]Diferric transferrin (310 nM) was added to all cells and samples were taken at intervals up to 3 h. After washing three times at 4 °C in PBS cells were lysed and total ^{59}Fe uptake was determined. (●) 1 mM-desferrioxamine without 5 mM-methylamine; (○) controls without 5 mM-methylamine; (■) 1 mM-desferrioxamine with 5 mM-methylamine; (□) controls with 5 mM-methylamine.

intracellular [^{59}Fe]ferrioxamine (Fig. 3) in accordance with cellular iron uptake, showing that transferrin continued to cycle in the presence of high concentrations of desferrioxamine. Exposure of cells to increasing concentrations of desferrioxamine resulted in increased chelator-bound counts, up to a concentration of 1 mM, when essentially all the iron entering the cell partitioned to ferrioxamine (Fig. 3). Higher concentrations of chelator (5 mM) did not lead to increased counts in this fraction (results not shown) since, under these conditions, there was excess intracellular chelator available to bind a limited amount of iron delivered to the cells by the transferrin endocytic cycle. Preincubation of cells for either 1 or 4 h with desferrioxamine did not alter the total counts associated with the chelator at each concentration of the drug.

Partition of intracellular ^{59}Fe to ferritin

In the presence of low concentrations of desferrioxamine (20–500 μM) a proportion (Fig. 2) of the iron delivered to the cells remained unbound by chelator. To determine whether this residual iron followed its anticipated route to the iron storage protein ferritin (Mattia *et al.*, 1986), [^{59}Fe]ferritin levels were measured in duplicate samples taken at the 3 h time point (Fig. 2). In control cells approx. 50% of the ^{59}Fe was incorporated into immunoprecipitable ferritin (Fig. 4). In the presence of increasing extracellular concentrations of desferrioxamine there was a decrease in the proportion of the counts found in ferritin with less than 5% of the iron following this pathway when the concentration was in

Fig. 7. Effect of methylamine on iron uptake and ferrioxamine formation in K562 cells

Conditions used were as described in the legend to Fig. 6. [^{59}Fe]Ferrioxamine was assayed by benzyl alcohol extraction at the 3 h time point only. □, Total ^{59}Fe ; ▨, ^{59}Fe -ferrioxamine.

excess of 1 mM. Clear differences were seen in the percentage of ferritin-bound ^{59}Fe between the 1 and 4 h preincubations. This is illustrated by the results with 50 μM - and 100 μM -desferrioxamine where there was a 30 to 40% decrease in immunoprecipitable ferritin counts after the prolonged exposure.

Release of [^{59}Fe]ferrioxamine

To investigate ferrioxamine release cells were preincubated with sufficient desferrioxamine (1 mM for 4 h) to ensure that all the ^{59}Fe donated to the cells by a 3 h incubation with [^{59}Fe]transferrin was chelated. Release of [^{59}Fe]ferrioxamine from the cells into chelator-free medium showed kinetics as demonstrated (Fig. 5) with 50% of the total loss occurring within 1.5–2 h. As the concentration gradient between intra- and extra-cellular ferrioxamine neared equilibrium, which was reached at around 24 h (results not shown), the rate of release slowed.

Chelation of transferrin iron during endocytosis

To examine whether iron was directly available to the chelator throughout the endocytic cycle or whether uncoupling from the protein was first required, cells were preincubated with the weak base methylamine, which prevents the release of iron from the two iron-binding sites of transferrin in K562 cells (Bomford *et al.*, 1985). Cells were loaded with 1 mM-desferrioxamine, preincuba-

ted with 5 mM-methylamine and the uptake of iron from [^{59}Fe]transferrin was measured over 3 h. Methylamine markedly reduced iron uptake by both the desferrioxamine-treated and control cells (Fig. 6) whereas in the absence of the base there was a linear uptake of iron. The formation of [^{59}Fe]ferrioxamine in cell lysates from the 3 h time point was inhibited by approx. 80% by methylamine (Fig. 7). Although, at this concentration of methylamine, cycling of the transferrin receptor in K562 cells is slowed to some extent (Bomford *et al.*, 1985; Ciechanover *et al.*, 1983), at least eight to ten rounds of endocytosis would be completed during the experimental period used here and it was concluded that uncoupling of iron from endocytosed transferrin was a prerequisite before this iron was in a chelatable form.

DISCUSSION

The majority of intracellular iron resides in protein-bound forms and two of these, transferrin and ferritin, have been extensively studied and well characterized. However, there appears to be a further biochemically indistinct pool of low-molecular-mass iron (Jacobs, 1977; Pollack & Campana, 1980) variously termed chelatable or labile (Jacobs, 1977). The concept has arisen that this pool is in equilibrium with iron donated to the cell by the transferrin cycle, with storage iron, in the form of ferritin, and with iron-dependent enzymes, some of which may have an important regulatory role in cellular iron metabolism. The functional significance of this pool would seem to be in a feed-back capacity by regulating the proteins that mediate the uptake (Testa *et al.*, 1985) and storage of iron (Rittling & Woodworth, 1984). When cells in culture are exposed to micromolar concentrations of desferrioxamine, leading to binding of iron within the pool, they respond by up-regulating transferrin receptors, an event which occurs after some hours (Bridges & Cudkowicz, 1984) and is probably under transcriptional control (Rao *et al.*, 1985). Conversely, iron supplementation, in the form of haemin (Rouault *et al.*, 1985) or diferric transferrin (Louache *et al.*, 1984) is thought to result in an enrichment of this pool and leads to receptor down-regulation. The level of chelatable iron within the cell has an analogous role in the regulation of ferritin synthesis (Rogers & Munro, 1987) through redistribution of mRNAs for the two protein subunits between the free pool and that bound to polyribosomes.

The location at which desferrioxamine exerts its effect and the sources of iron available for chelation are poorly characterized. Previous studies using cultured hepatocytes (Octave *et al.*, 1983) and Chang cells (White *et al.*, 1976b) have focused on ferritin as the major source of chelatable iron. Although in the intact animal model it has been demonstrated that ^{59}Fe donated to hepatocytes by transferrin is effectively chelated (Pippard *et al.*, 1982) the mechanisms involved were not investigated. In view of the abundant (Klausner *et al.*, 1983) and closely regulated (Louache *et al.*, 1984) numbers of transferrin receptors on K562 cultured erythroleukaemia cells it can be anticipated that the transferrin cycle could provide a dynamic and potentially rich source of iron for an intracellular chelating agent.

We initially investigated the rate at which desferrioxamine entered K562 cells using an indirect method in which the conversion of repeated pulses of [^{59}Fe]-

transferrin to [^{59}Fe]ferrioxamine was determined. Maximal conversion of the pulse occurred when the cells had been exposed to desferrioxamine for 40–60 min suggesting that by this time equilibrium had been reached. These results are in close accord with those of Laub *et al.* (1985), who found that the uptake into cultured hepatocytes of derivatized forms of desferrioxamine reached a plateau after 60 min and that the total uptake was proportional to the extracellular concentration of the compound. These results contrast with the evidence of Bottomley *et al.* (1985), who investigated directly the uptake of a ^{14}C -labelled preparation of the chelator in K562 cells. They found continued uptake of the label over a 24 h period, when cells were incubated in $20\text{ }\mu\text{M}$ - ^{14}C desferrioxamine with a resultant 14-fold accumulation of ^{14}C within the cell. We have also observed (S. Roberts & A. Bomford, unpublished results) linear uptake of [^{14}C]desferrioxamine by K562 cells with a 6–20-fold accumulation of the label when extracellular concentrations of between $100\text{ }\mu\text{M}$ and 1 mM were used. However, because of the evidence that there is extensive degradation of the drug in mammalian plasma and a degree of cellular metabolism by cultured hepatocytes (Laub *et al.*, 1985) these results are difficult to interpret since intracellular ^{14}C is not a true reflection of the level of intact and functioning desferrioxamine. This problem is avoided in the present study because we assessed the intracellular chelating capacity at a given extracellular concentration and did not attempt to determine the actual intracellular level of desferrioxamine.

At all concentrations of desferrioxamine, [^{59}Fe]ferrioxamine was rapidly generated, indicating that there was immediate chelation of transferrin-derived iron, although over the first 10 min of the incubation ligand–receptor cycling, iron release from transferrin and passage to the cytosol appeared to limit the initial rate of ferrioxamine formation. In the range $20\text{--}500\text{ }\mu\text{M}$ the partitioning of iron to chelator was wholly dependent on the extracellular concentration of desferrioxamine, provided the cells had been preincubated with the drug for longer than 60 min. Given that the effective intracellular chelating capacity rose with increased extracellular concentration we propose that the proportion of iron bound is a reflection of the amount of desferrioxamine within the cell. The partitioning of iron to chelator may also be dependent upon the relative affinities of desferrioxamine and any endogenous iron-acceptor molecule, which if it is to compete for ferric iron must have an affinity constant in the order of 10^{31} . At concentrations greater than 1 mM when intracellular levels of chelator were in excess of iron delivery (which was dependent upon the rate of endocytosis), there was complete conversion of iron to ferrioxamine. This again contrasts with previous data from Bottomley *et al.* (1985) who reported that only $40\text{ }\mu\text{M}$ -desferrioxamine led to 86% [^{59}Fe]ferrioxamine formation as assessed by co-elution of cell lysate with a standard on a Sephadex G-50 column. It is difficult to understand the 20-fold discrepancy between their results and those of the present study. An explanation may lie in the 24 h preincubation of cells with chelator used by Bottomley *et al.* (1985). This would cause increased iron delivery through up-regulation of transferrin receptors (Bridges & Cudkowicz, 1984; Mattia *et al.*, 1984) and decreased iron storage capacity through a reduction in ferritin protein synthesis (Rittling & Woodworth, 1984) which may be sufficient to

explain the increased partitioning of entering iron to chelator. Our results also indicate that an equilibrium exists between the entry of desferrioxamine into cells, the generation of the iron loaded form and the release of ferrioxamine from the cells since once maximum intracellular chelating concentrations have been reached there is no decline in the fraction of iron following this pathway.

If, however, intracellular desferrioxamine concentrations are insufficient to bind all the iron entering the cells the residual iron passes to the storage protein ferritin, so the possibility exists that after continued incubation, chelator may remove iron from this source. Our preliminary results (not shown) suggest that for up to 4 h after binding by ferritin, iron may be accessible to desferrioxamine. A recent report (Mattia *et al.*, 1986) has indicated that iron liberated from transferrin can reside within the cell for 2–5 h before becoming incorporated into ferritin. We suggest that desferrioxamine interrupts the flow of iron into this pre-ferritin compartment and may bind iron directly while it remains loosely associated with the molecule.

The prolonged incubation of cells with desferrioxamine affected the partition of iron between ferrioxamine and ferritin in an additional way. The translation of ferritin mRNA is sensitive through cytoplasmic control (Zahringer *et al.*, 1976) to levels of chelatable iron within the cell (Rogers & Munro, 1987) so that changes in ferritin protein synthesis are effected in a matter of 4–6 h (Drysdale & Munro, 1966). Mattia *et al.* (1986) have proposed that the regulation of intracellular iron distribution is dependent upon absolute ferritin levels and is unaffected over a wide range of concentrations of iron delivered to the cell. Our results lend support to this theory. Cells which were incubated with desferrioxamine for a total of 7 h had levels of intracellular chelator identical with those incubated for a total of 4 h (Fig. 2), but the fraction of the total cellular iron partitioning to ferritin was reduced by between 30–35% after the longer exposure (Fig. 4) which may correlate with the expected reduction in the level of ferritin protein.

We have also shown that desferrioxamine can only bind transferrin iron after it has been released from the endocytosed protein, suggesting that the chelator is competing with an endogenous acceptor molecule within either the endosome, the endosomal membrane or in the cytosol. Neutralization of intravesicular pH with methylamine which leads to the recycling of the diferric species (Bomford *et al.*, 1985) prevented the release of iron to desferrioxamine and markedly reduced ferrioxamine formation. Whether desferrioxamine exerts its action within the endosome or at a cytosolic location is unclear. This chelating agent enters lysosomes (Laub *et al.*, 1985) and possibly other types of intracellular vesicle, but since the transferrin-containing endosome has a residency time of only 5–10 min in K562 cells (Klausner *et al.*, 1983; Bomford *et al.*, 1985) it would seem unlikely that effective concentrations of the chelator could accumulate within the vesicle during this period. In addition the half-time for ferrioxamine release (1.5–2 h) was considerably longer than would be expected if the compound was released directly from the endosome during exocytosis.

We have established that the entry of transferrin iron into a chelatable pool is limited by the rate of the endocytic cycle. Presumably the number of transferrin

receptors also limits intracellular chelatable iron levels. It will be of interest to examine the partitioning of iron to desferrioxamine in a variety of cell types exhibiting a range of transferrin receptor numbers. Further work is also required to determine whether under conditions of iron deprivation or supplementation it is the size of the chelatable pool that is altered or the rate of iron passage through it.

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