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Assay and Characteristics of the Iron Binding Moiety of Reticulocyte Endocytic Vesicles

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Summary. A ^{59}Fe assay was designed to detect an Fe(III) binding capacity in NP-40 solubilized proteins from rabbit reticulocyte endocytic vesicles. The iron binding capacity had an apparent molecular weight as determined by gel exclusion chromatography of 450,000 daltons. The iron binding moiety coincided with the major nontransferrin iron-containing material of endocytic vesicles labeled in vivo by incubation of cells with ^{59}Fe, ^{125}I-labeled transferrin. The material solubilized from vesicles with NP-40 exhibited two classes of saturable binding sites, one with an association constant for ^{59}Fe-citrate of 3.63×10^9 M^{-1} and with 6.6×10^{-12} moles of iron bound per mg protein and the other with a constant of 3.96×10^8 M^{-1} and 1.0×10^{-12} moles of iron bound per mg protein. These affinities are sufficient to satisfy the solubility characteristics of Fe(III) at pH 5.0. Most of the ^{59}Fe bound both in vivo and in vitro to the iron binding moiety could be displaced with ^{56}Fe and an equivalent amount of ^{59}Fe could subsequently be rebound in vitro. The iron binding assay was adopted to vesicle proteins separated by SDS-polyacrylamide gel electrophoresis with subsequent transfer to nitrocellulose and revealed an iron binding activity of molecular weight approximately 95,000 daltons.

Key Words endocytic vesicles · iron

Introduction

Iron uptake in erythroid cells (Jandl & Katz, 1963; Iacopetta & Morgan, 1983) and other mammalian cells (van Renswounde et al., 1982; Enns et al., 1983) is initiated by the binding of iron-loaded transferrin to transferrin receptors located on the cell surface. The transferrin-receptor complex is then internalized by a process of receptor-mediated endocytosis (Young & Aisen, 1980; Karin & Mintz, 1981; Octave et al., 1982; Enns et al., 1983; Iacopetta & Morgan, 1983; Nunez & Glass, 1983), and iron is released from transferrin by the acid millieu within the endocytic vesicle (van Renswounde et al., 1982; Armstrong & Morgan, 1983; Nunez & Glass, 1985; Bakkeren et al., 1987). Once iron loses affinity for and detaches from transferrin, the iron still has to cross the barrier of the vesicle membrane before reaching the cytosol iron carriers for transport to the mitochondria. Evidence indicates that the released Fe(III) is transiently bound to a plasma membrane protein (Speyer & Fielding, 1974; Glass, Nunez & Robinson 1980) and then reduced to Fe(II) (Nunez, Cole & Glass, 1983) prior to mobilization by cytosol carriers.

The nature of the moiety to which iron binds is unknown, and its identification has been hindered by the complications of the chemistry of iron in aqueous solutions. Knowledge of the molecular characteristics of the moiety could help to better characterize the subsequent transport of iron through the vesicle membrane. This paper reports the development of an assay method for the iron binding moiety in rabbit reticulocyte endocytic vesicles carrying the transferrin-transferrin receptor complex. This assay has allowed the initial characterization of the iron binding moiety including determination of the affinity binding constant for iron and the apparent molecular weight of the moiety.

Materials and Methods

Rabbit reticulocytes were obtained from animals responding to 1-acetyl-2- phenylhydrazine (Glass et al., 1980). Cells were used from day 4 and day 6 after the last injection of the drug. Rabbit transferrin was purified and labeled with ^{59}Fe and then with ^{125}I as described previously (Martinez-Medellin & Shulman, 1972). To eliminate traces of iron, all reagents used were filtered through Chelex-100 resin (Bio-Rad).

Rabbit reticulocyte endocytic vesicles containing the transferrin-receptor complex were prepared by differential centrifugation (Choe et al., 1987). Vesicles, at a concentration of 2 mg protein/ml, were solubilized with 5 mg/ml of Nonidet P-40 (Sigma Chemical Co.). Solubilized proteins were separated from residual proteins by centrifugation for 10 min at $10,000 \times g$.

Measurement of the Iron Binding Capacity

The method designed to measure the iron binding capacity took into account the chemical characteristics of Fe(III) in solution as

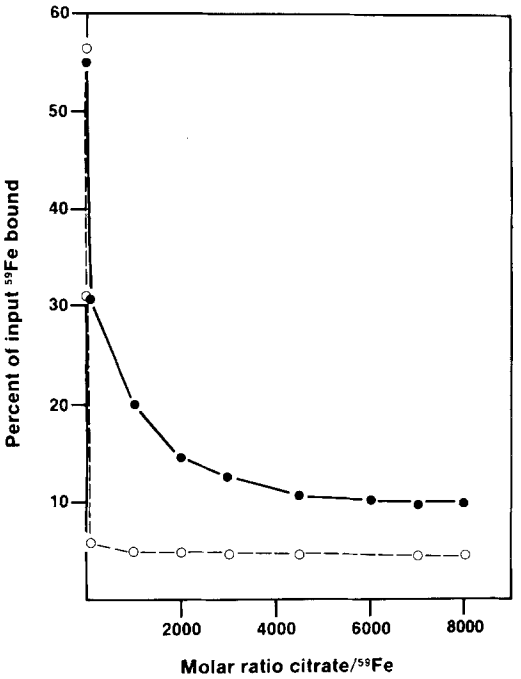

Fig. 1. Binding of ^{59}Fe to solubilized vesicles as a function of iron : citrate molar ratio. Endocytic vesicles (2 mg/ml) were solubilized with Nonidet P-40 and the solubilized material was incubated with 60 nM ^{59}Fe with different concentrations of citrate in a medium that was 100 mM Na acetate (pH 5.0). The reaction was stopped with cold acid-ethanol as described in Materials and Methods and radioactivity determined in the uniformed fraction (supernatant) and bound fraction (pellet). Shown is the percent of input ^{59}Fe bound in the presence (●) and in the absence (○) of vesicle proteins

well as the conditions in which iron would be released from transferrin inside the endocytic vesicle (van Renswounde et al., 1982; Armstrong & Morgan, 1983). Nanomolar amounts of iron as a ^{59}Fe(III)-citrate complex with a 5000-M excess of citrate was added to a solubilizate of vesicle proteins in a medium buffered with 0.1 M acetate (pH 5.0). The molar ratio of Fe to citrate was found to effect a low, reversible binding of ^{59}Fe with minimal background precipitation of ^{59}Fe-citrate when, in the course of the assay, the bound isotope was separated from the free isotope. A pH of 5 was *a priori* selected for the incubation medium in an intent to mimic the intravesicular pH (van Renswounde et al., 1982; Armstrong & Morgan, 1983). Further, it was unlikely that polynuclear iron complexes would form under these conditions (Spiro, Bates & Saltman 1967). Acetate buffer was shown not to interfere either with the binding of iron to membrane components or the further ethanol precipitation of the protein. Separation of free and bound iron was achieved by precipitation of the bound iron with ice-cold acid ethanol (65% final ethanol concentration, (pH 5.0)) in the presence of 100 μg of carrier bovine serum albumin. After centrifugation for 10 min at 5,000 × g, supernatant and pellet were carefully separated. Residual supernatant was eliminated by carefully wiping of the interior wall of the tubes. Washing of the ethanol precipitate was avoided because of the reversibility of the binding reaction. ^{59}Fe radioactivity in the supernatant and pellet was determined and interpreted as estimates of the free and bound ^{59}Fe, respectively. The binding reaction was carried out in clear polyethylene tubes (Sarstedt Co.), which showed low background absorption of ^{59}Fe. Nonspecific binding was determined in parallel tubes using a 100-fold excess of ^{56}Fe. An alternative method for separating bound from unbound ^{59}Fe was used for determining ^{59}Fe bind to solubilized vesicles as a function of increasing ^{59}Fe concentration. In this procedure the acid-ethanol precipitate was filtered through 0.22 μm Millipore GSWP filters prewashed with 0.1 mM $FeCl_3$-500 mM Na citrate, pH 5.0, and radioactivity retained by the filters determined without further washing.

Detection of Iron Binding Protein after SDS-Polyacrylamide Gel Electrophoresis

Aliquots of vesicles were separated by SDS-polyacrylamide gel electrophoresis (Laemmeli, 1970) and proteins transferred to nitrocellulose paper using a transfer buffer of 190 mM glycine, 10 mM Tris-HCl, 20% methanol (pH 8.3). The nitrocellulose paper was blocked with 1% bovine serum albumin in 100 mM NaCl, washed with 5 mM Na citrate, 100 mM NaCl (pH 5.0), reacted at 37°C for 30 min with 10 nM $^{59}FeCl_3$, 50 mM Na citrate, 100 mM NaCl (pH 5.0), washed extensively with 200 mM Na acetate (pH 5.0), and binding of ^{59}Fe detected by radioautography.

Results

The Assay Binding of ^{59}Fe(III) to Vesicle Proteins

The iron binding capacity of NP-40 solubilized vesicles was evident when reacted with ^{59}Fe(III)-citrate at pH 5.0 (Fig. 1). Molar ratios of citrate to iron between 4,000 and 10,000 produced a constant level of bound iron (Fig. 1). With ratios less than 4,000, increased ^{59}Fe binding was observed. In subsequent assays a citrate : iron ratio of 5,000 was employed. A ^{59}Fe binding capacity similar to the constant level shown in Fig. 1 could be obtained using nitrilotriacetate-^{59}Fe complexes at a molar ratio of 50 : 1. DTPA-^{59}Fe and EDTA-^{59}Fe complexes were unable to donate iron to the solubilized proteins, presumably because of the higher affinity of the latter two chelators for iron as compared with the affinity of the membrane iron binding moiety (*see below*). Under the conditions of the assay, no binding of ^{59}Fe to transferrin alone could be demonstrated.

When increasing concentrations of ^{59}Fe-citrate were added to detergent-solubilized vesicle proteins a saturable iron binding capacity in the nanomolar range was found (Fig. 2*a*). Scatchard analysis of the binding data demonstrated (Fig. 2*b*) two binding components with apparent association constants of $2.8 \pm 1.1 \times 10^8$ M^{-1} and $3.1 \pm 1.0 \times 10^7$ M^{-1} (mean ± SD of three experiments). The binding capacity was $6.6 \pm 2.1 \times 10^{-12}$ and $1.0 \pm 0.5 \times 10^{-11}$ moles iron bound per mg protein (means ± SD of three

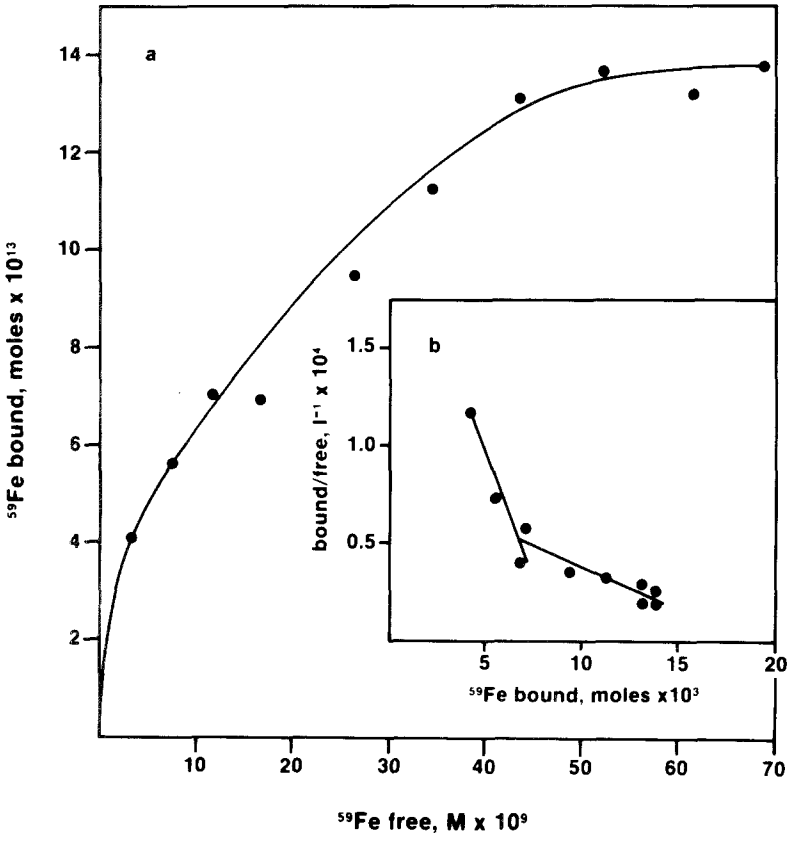

Fig. 2. Binding analysis of ^{59}Fe to solubilized vesicle proteins. Solubilized endocytic vesicles (100 μg/tube) were incubated with increasing concentration of ^{59}Fe-citrate, precipitated and collected on Millipore filters as described in Materials and Methods. (*a*) Binding of ^{59}Fe to vesicle proteins at increasing ^{59}Fe concentrations. (*b*) Scatchard analysis of *a*

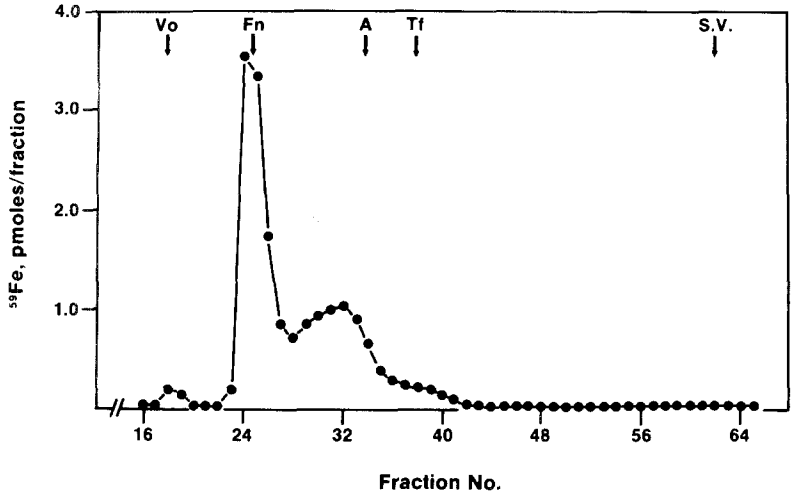

Fig. 3. Gel permeation fractionation of ^{59}Fe-labeled vesicles. Cells were incubated with ^{59}Fe, ^{125}I-labeled transferrin, vesicles prepared and solubilized as described. The detergent solubilized proteins from 0.5 of cells were then fractionated on Bio-Gel A-1.5, equilibrated with 20 mM HEPES (pH 7.4)-5 mg/ml Nonidet P-40. Shown is the ^{59}Fe radioactivity in the eluate. The column was calibrated with blue dextran (*Vo*), ferritin (*Fn*), and transferrin (*Tf*)

experiments). The binding of ^{59}Fe could be entirely competed for with ^{56}Fe.

Characterization of the Iron Binding Moiety

A partial characterization of the iron binding component was achieved by Bio-Gel A-1.5 gel permeation chromatography of the solubilized vesicles (Fig. 3). The gel permeation pattern of solubilized vesicle proteins derived from cells previously incubated with ^{59}Fe, ^{125}I-transferrin show two prominent peaks of ^{59}Fe radioactivity. The first peak (fractions 25–28) of apparent molecular weight 450,000 daltons had no associated ^{125}I-transferrin. We have previously shown that this material did not react with antibodies to rabbit reticulocyte ferritin (Glass

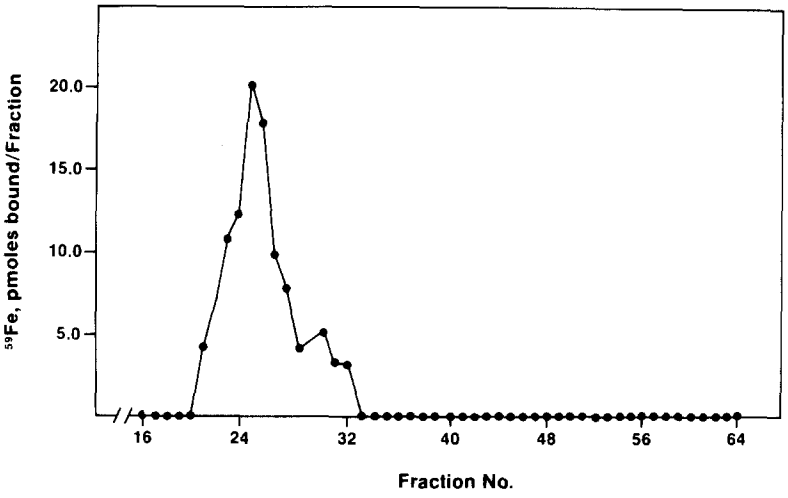

Fig. 4. Iron binding capacity of gel permeation fractionated vesicles. Endocytic vesicles from 2 ml of cells were solubilized with Nonidet P-40 and then fractionated through a column of Bio-Gel A-1.5 as described for Fig. 3. Collected fractions were assayed for iron binding capacity as described in Materials and Methods

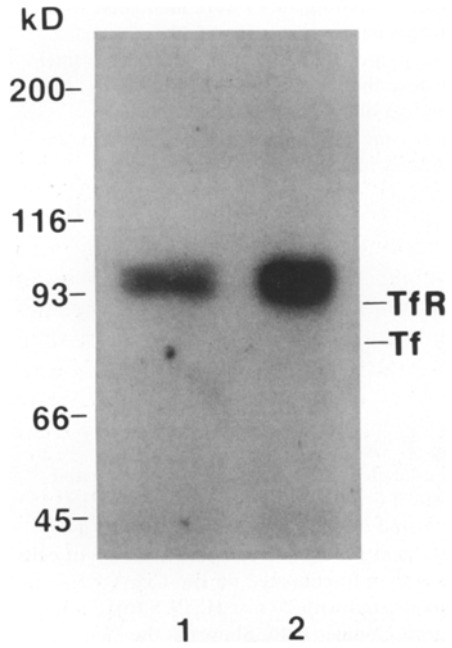

Fig. 5. Iron binding capacity of SDS-polyacrylamide gel electrophoresis fractionated vesicle proteins. Fifty μg of protein, either of reticulocyte stroma (lane *1*) or vesicles (lane *2*), were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with ^{59}Fe citrate as described in Materials and Methods. Molecular weights were determined from the following standards detected by Coomassie blue staining: (i) myosin 205,000 mol wt, (ii) β-galactosidase 116,500 mol wt, (iii) phosphorylase B 97,400 mol wt, (iv) bovine serum albumin 66,000 mol wt, (v) egg albumin 45,000 mol wt. The location of transferrin (*Tf*) and transferrin receptor (*TfR*) was determined by Coomassie blue staining of a parallel lane of vesicles

et al., 1980). The second peak with an apparent molecular weight of 300,000 daltons comigrates with the major transferrin peak that corresponds to the transferrin-transferrin receptor complex (Glass et al., 1980). No other peaks of ^{59}Fe radioactivity were evident under these conditions.

It was of interest to ascertain if unlabeled solubilized vesicle proteins subjected to gel filtration had an iron binding capacity with molecular weight similar to that seen with in vivo labeling. Vesicles obtained from unlabeled cells were solubilized and gel filtered as above and the column eluent was assayed for iron binding capacity. An iron binding capacity with a peak activity of about 20.0 picomoles of ^{59}Fe bound/fraction was found in the 450,000 mol wt region (Fig. 4). The region corresponding to the transferrin-receptor complex showed a much lower iron binding capacity.

Further characterization of the iron binding capacity was achieved by subjecting solubilized vesicle proteins to SDS-polyacrylamide gel electrophoresis and assaying the separated proteins for iron binding capacity. In this assay the proteins were transferred to nitrocellulose paper and the paper was incubated with ^{59}Fe-citrate as described in Materials and Methods. A major ^{59}Fe binding activity was found with an apparent molecular weight of about 95,000 daltons (Fig. 5). Densitrometric tracings of radioautographs demonstrated more ^{59}Fe binding activity in vesicle proteins than equivalent amounts of reticulocyte plasma membrane proteins separated by SDS-polyacrylamide gel electrophoresis. Similar ^{59}Fe binding was seen if solubilized vesicles were first separated by gel permeation chromatography and the high molecular weight iron binding peak then subjected to electrophoresis, transferred to nitrocellulose, and incubated with ^{59}Fe-citrate. Using parallel lanes of vesicles stained with Coomassie blue it was possible to distinguish the iron binding material as distinct from transferrin and the transferrin receptor. Further, under the condition of the assay, no binding was observed of

^{59}Fe either to transferrin or the transferrin receptor.

To determine if the iron binding characteristics of the protein(s) labeled by the in vitro assay were similar to those of the protein(s) labeled in vivo, the following experiment was performed. The high molecular weight peak from gel permeation chromatography was obtained either from vesicles prepared from cells preincubated with ^{59}Fe, ^{125}I-labeled transferrin ("in vivo" ^{59}Fe-labeled peak) or from unlabeled vesicles. The peak from unlabeled cells was then labeled by incubation with 60 nM ^{59}Fe-citrate ("in vitro" ^{59}Fe-labeled peak). The in vivo and in vitro bound ^{59}Fe was then subjected to displacement by ^{56}Fe-citrate. Similar displacements of about 80% was seen with both activities (Table). Subsequently the in vivo-labeled material after displacement with ^{56}Fe was relabeled with ^{59}Fe-citrate. The amount of iron incorporated was similar to that originally found in the in vivo labeled peak (Table 1).

We have previously shown an enrichment of ^{59}Fe in a high molecular weight iron binding moiety from reticulocyte plasma membranes incubated with isoniazid and ^{59}Fe-transferrin (Glass et al., 1980). Consistent with this observation, a quantitatively different picture from that described above was seen with vesicles from reticulocytes treated with isoniazid and extensively labeled with ^{59}Fe-transferrin. The high molecular weight ^{59}Fe containing material from solubilized vesicles prepared from these isoniazid-treated cells showed about 67.7×10^{-11} moles of iron/mg protein. While only about 17.2×10^{-11} moles of iron could be displaced with ^{56}Fe, 19.4×10^{-11} moles could be rebound when reincubated with ^{59}Fe-citrate.

Table. The binding and displacement of ^{59}Fe to in vivo and in vitro labeled proteins[a]

	Binding (moles iron/mg protein)	Displacement (%)
in vivo labeled	8.6 ± 10^{-12}	77.2
in vivo relabeled	19.4 ± 10^{-12}	—
in vitro labeled	19.6 ± 10^{-12}	79.2

[a] The ^{59}Fe-labeled high molecular weight peak prepared from gel permeation chromatography of vesicles from in vivo-labeled reticulocytes prepared as described in Materials and Methods was incubated with 80 nM ^{56}Fe as in the iron binding assay, precipitated with acid-ethanol, radioactivity determined, resuspended and incubated with 80 nM ^{59}Fe (in vivo relabeled). In a parallel experiment, the high molecular weight moiety from gel permeation chromatography of solubilized vesicles was incubated first with ^{59}Fe (in vitro labeled), radioactivity determined, the precipitate resuspended and ^{59}Fe displaced by incubation with ^{56}Fe.

Discussion

Recently we have proposed that iron crosses the endocytic vesicle membrane by a transporter-mediated process in which iron is translocated from the *cis* or intravesicular side to the *trans* or cytosolic side by a transporter located in the vesicle membrane (Nunez & Glass, 1985). The first step in this transport requires iron released from transferrin to bind to a membrane iron binding moiety. The iron binding assay described detects an iron binding moiety with an apparent high molecular weight in reticulocyte endocytic vesicles solubilized with a nonionic detergent. The moiety appears to have two classes of iron binding sites as distinguished by two different affinities for ^{59}Fe(III). The presence of two iron binding components in solubilized vesicle proteins could result either from (i) two different proteins with different affinities for iron, or (ii) one protein with two binding sites for iron. At the present neither of the alternatives can be distinguished. The components labeled with ^{59}Fe in vitro have the same apparent molecular weight as the in vivo ^{59}Fe-labeled moiety as estimated by gel permeation chromatography. Both the in vivo and in vitro ^{59}Fe-labeled material have the capacity for ^{59}Fe to be displaced by ^{56}Fe and to be relabeled to the same extent with ^{59}Fe, indicating that the in vitro-labeled sites are the functional, physiologic sites in endocytic vesicles. It is interesting that with isoniazid, which inhibits iron translocation into the cytosol (Glass et al., 1980), the iron binding capacity of the vesicle is increased more than sixfold. This preliminary observation suggests that cellular iron status can regulate the iron binding capacity of the vesicles. The apparent affinity constant of iron for the iron binding component is the result of the relative affinities of iron for citrate and for the iron binding component, which in a simplified form can be estimated as follows:

$$\text{Fe} + \text{citrate} \rightarrow \text{Fe-citrate} \qquad K_1 = \frac{[\text{Fe-citrate}]}{[\text{Fe}][\text{citrate}]} \tag{1}$$

$$\text{Fe-citrate} + \text{IBC} \rightarrow \text{Fe-IBC} + \text{citrate} \qquad K_2 = \frac{[\text{Fe-IBC}][\text{citrate}]}{[\text{Fe-citrate}][\text{IBC}]} \tag{2}$$

$$\text{Fe} + \text{IBC} \rightarrow \text{Fe-IBC} \qquad K_3 = \frac{[\text{Fe-IBC}]}{[\text{Fe}][\text{IBC}]} = K_1 \cdot K_2 \tag{3}$$

where IBC represents the iron binding component and K_1, K_2 and K_3 are the association constants for

the reactions described. (For simplicity no charges are considered.) As discussed by Aisen, Leibman and Zweier (1978) the interaction of Fe(III) with citrate is far more complex than indicated above, involving the formation of ferric monocitrate and ferric dicitrate. Utilizing the equilibrium constants for the various interactions given by Aisen et al. (1978) and Spiro et al. (1967), the experimentally determined association constants of ^{59}Fe(III) for the iron binding component of 2.83×10^7 and 3.1×10^7 M^{-1} can be corrected to 3.63×10^9 and 3.96×10^8 M^{-1}, respectively. Further, it can be calculated that under the conditions of the assay the predominant ion donating species by many orders of magnitude would be ferric monocitrate rather than the dicitrate species or free ferric ions.

The maximal concentration of Fe(III) in solution is given by the solubility product of $Fe(OH)_3$. At pH 5, a likely pH for the inside of the endocytic vesicle (van Renswounde et al., 1982; Paterson et al., 1984), the maximal concentration of free Fe(III) is about 10^{-10} M. Hence, inside a functional endocytic vesicle, free iron concentrations should be expected to be lower than 10^{-10} M. The affinity constant of the IBP will be sufficient to allow the maximal amount of free Fe(III) at pH 5 as long as there are three free IBPs available for every one Fe-IBP complex. That the iron binding capacity can be increased markedly by treatment of cells with isoniazid suggests that there is "excess" iron binding capacity. The condition can also be assisted if the system is rapidly drained by the translocation process. This situation is likely to exist as the rate constant for translocation of iron to cytosol is fourfold greater than the rate constant for iron dissociation from transferrin (Nunez & Glass, 1988[1]). The solubility of dissociated iron will be highly dependent on the pH of the vesicle. For example, an increase of the intravesicular pH to pH 5.5 will require that for each Fe-IBP complex, there be about 150 free IBPs, a condition which may still be fulfilled by the observed differences in the rate constants and by the excess iron binding capacity. However, at pH 6.0 to maintain iron solubility would require an excess of nearly 3,000 free IBPs. This requirement is consistent with the observations that (i) concentrations of lysosomotropic agents with slight effects on intravesicular pH nonetheless inhibit iron uptake (Paterson et al., 1984; Nunez & Glass, 1985); and (ii) that the delay in iron release from internalized transferrin may correlate with the time to full acidification of the endosomes (Bakkeren et al., 1987).

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