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Kinetics of Iron Passage through Subcellular Compartments of Rabbit Reticulocytes

J. Abra Watkins†, Marco-Tulio Nunez, Victoria Gaete, Osvaldo Alvarez, and Jonathan Glass†
Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile, and †Department of Medicine, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Summary. The kinetics of the separate processes of Fe_2(III)-transferrin binding to the transferrin receptor, transferrin-receptor internalization, iron dissociation from transferrin, iron passage through the membrane, and iron mobilization into the cytoplasm were studied by pulse-chase experiments using rabbit reticulocytes and ^{59}Fe, ^{125}I-labeled rabbit transferrin. The binding of ^{59}Fe-transferrin to transferrin receptors was rapid with an apparent rate constant of 2×10^5 M^{-1} sec^{-1}. The rate of internalization of ^{59}Fe-transferrin was directly measured at 520 ± 100 molecules of Fe_2(III)-transferrin internalized/sec/cell with 250 ± 43 sec needed to internalize the entire complement of reticulocyte transferrin receptors. Subsequent to Fe_2(III)-transferrin internalization the flux of ^{59}Fe was followed through three compartments: internalized transferrin, membrane, and cytosol.

A process preceding iron dissociation from transferrin and a reaction involving membrane-associated iron required 17 ± 2 sec and 34 ± 5 sec, respectively. Apparent rate constants of 0.0075 ± 0.002 sec^{-1} and 0.0343 ± 0.0118 sec^{-1} were obtained for iron dissociation from transferrin and iron mobilization into the cytosol, respectively. Iron dissociation from transferrin is the rate-limiting step. An apparent rate constant of 0.0112 ± 0.0025 sec^{-1} was obtained for processes involving iron transport through the membrane although at least two reactions are likely to be involved. Based on mechanistic considerations, iron transport through the membrane may be attributed to an iron reduction step followed by a translocation step. These data indicate that the uptake of iron in reticulocytes is a sequential process, with steps after the internalization of Fe_2(III)-transferrin that are distinct from the handling of transferrin.

Key Words iron uptake · transferrin · endocytosis · reticulocytes · iron mobilization · iron translocation

Introduction

The cellular process of iron uptake is initiated with the binding of iron-loaded transferrin to transferrin receptors on the surface of the cell (Jandl & Katz, 1963). The transferrin-transferrin receptor complex is then internalized by a process of adsorptive endocytosis (Harding, Heuser & Stahl, 1983; Iacopetta & Morgan, 1983). This process commences with invagination of a clathrin-coated pit containing the transferrin-transferrin receptor complex. During the endocytic process, the resulting coated vesicle loses its clathrin coat and undergoes acidification by a H^+-ATPase which allows Fe(III) to dissociate from transferrin. The released Fe(III) is reduced to Fe(II), binds to a membrane moiety (Nunez, Pinto & Glass, 1989), and then crosses the endocytic vesicle membrane for release to the cytosol (Nunez & Glass, 1983, 1985). Because of acidification, apo-transferrin remains bound to the transferrin receptor and the complex undergoes exocytosis by vesicle-plasma membrane fusion leading to apo-transferrin release and return of transferrin receptors to the cell surface (van Renswoude et al., 1982; Dickson et al., 1983; Harding et al., 1983; Lamb et al., 1983; Hanover, Willingham & Pastan, 1984). Although kinetic models describing the endocytic cycle of transferrin have been reported (Ciechanover et al., 1983; Iacopetta & Morgan, 1983), details of the specific processes involved in iron transport remain unclear. The aim of the present work is to define the events associated with iron uptake, as distinct from transferrin handling, and to present a testable molecular hypothesis for iron transport across the vesicle membrane.

Based on observations of iron internalization, the general events of the iron uptake process can be formulated as follows:

Binding:
(1) $Fe_2(III)\text{-Tf} + R \rightarrow [Fe_2(III)\text{Tf-}R]_{out}$
Internalization:
(2) $[Fe_2(III)\text{Tf-}R]_{out} \rightarrow [Fe_2(III)\text{Tf-}R]_{in}$
Dissociation:
(3) $IBP + [Fe_2(III)\text{Tf-}R]_{in} \rightarrow R\text{-Tf} + [IBP\text{-}Fe_2(III)]_{cis}$
Mobilization:
(4) $[IBP\text{-}Fe_2(III)]_{cis} + C_{trans} \rightarrow IBP + (C\text{-}Fe(II))_{trans}$.

In this sequence, cellular iron uptake is initiated

with the binding of loaded diferrictransferrin (Fe $(III)_2$-Tf) to transferrin receptors (*R*) on the reticulocyte surface (Reaction 1) followed by internalization (Reaction 2) of the $Fe(III)_2$Tf-*R* complex (van Renswoude et al., 1982; Dickson et al., 1983; Enns et al., 1983; Harding et al., 1983; Iacopetta & Morgan, 1983; Lamb et al., 1983; Hanover et al., 1984). Iron dissociation from transferrin and transfer to an iron-binding membrane protein (IBP; Nunez et al., 1989) as shown by Reaction 3, involves acidification of endocytic vesicles (Speyer & Fielding, 1974; Glass, Nunez & Robinson, 1980; Nunez & Glass, 1983). Mobilization of iron by cytoplasmic carriers (*C*) follows processes involving iron reduction to Fe(II) and translocation from the *cis* (or intralumenal) to the *trans* (or cytosolic) side of the vesicle membrane (Nunez, Glass & Cole, 1980; Pollack, Campana & Weaver, 1985; Egyed, 1988; Morgan, 1988; Sturrock, et al., 1990). The steps following internalization (Reactions 3 and 4) are separate from transferrin endocytosis and unique to the intracellular handling of iron.

A detailed kinetic description of iron uptake using pulse-chase methods for measurement of ^{59}Fe turnover in rabbit reticulocytes and characteristic times for internalization of transferrin, iron binding to IBP, and iron appearance in the cytosol has been derived following consideration of several possible reaction schemes. The results demonstrate that iron dissociation from transferrin is the rate-limiting step and is followed by a minimum of two reactions in the membrane before iron can be mobilized to the cytoplasm. Further, a considerable portion of the transferrin cell cycle is required for complete delivery of iron to the cytoplasm. Rate constants describing iron dissociation from transferrin, iron transport through the membrane, and iron mobilization into the cytosol are computed for several models.

Materials and Methods

Cells and Transferrin

Reticulocytes were obtained from rabbits by 1-acetyl-2-phenyl hydrazine treatment and phlebotomy (Glass et al., 1980). Rabbit transferrin was purified from plasma by a modification of the method described by Martinez-Medellin and Schulman (1972). The purified transferrin was saturated with iron using ^{59}Fe (Nunez, Cole & Glass, 1983*a*) and then labeled with ^{125}I by the iodine monochloride method (Contreras, Bale & Spar, 1983). ^{59}Fe was purchased from New England Nuclear (Boston, MA), and ^{125}I from Commission de Energia Nuclear (Santiago, Chile) or New England Nuclear.

Determination of Surface and Total Number of Transferrin Receptors and of the Transferrin Cell Cycle Time

In order to express the rate of ^{59}Fe transport through the various iron compartments as a function of the total transferrin cycle time it was necessary to determine the total number of transferrin receptors, the number of receptors on the surface of the reticulocytes and the transferrin cell cycle time. The total number of transferrin receptors was determined as described (Iacopetta & Morgan, 1983). The fraction of the total receptors on the surface of the cells was determined by incubation of 40 μl of packed cells for 20 min on ice with 60 μl of ^{59}Fe, ^{125}I-labeled transferrin (final concentration 1.5 μM) in Dulbecco's saline made 10 mg/ml in bovine serum albumin (Dulbecco's-BSA). The mixture was then centrifuged through 0.5 ml of dibutylphtalate (Sigma, St. Louis, MO) in a microcentrifuge at 8,000 rpm for 20 sec, and the cells were incubated for 60 min on ice with 200 μl of rabbit plasma. After centrifugation, the ^{125}I radioactivity associated with the supernatant was determined in a two-channel Phillips gamma counter. Preliminary experiments indicated that the incubation with plasma produced complete displacement of specific, surface-bound transferrin.

The transferrin cycle time was defined as the average time taken for a receptor to bind Fe-transferrin, internalize the Fe-transferrin into the cell, and then externalize and release the apo-transferrin. The transferrin cell cycle time was estimated as follows:

$$\text{transferrin cell cycle time} = \frac{t_0}{R(\text{Fe/I})_{\text{cell}}/R(\text{Fe/I})_{\text{Tf}}}.$$

Where t_0 is the time of incubation of cells with ^{59}Fe, ^{125}I-labeled transferrin, $R(\text{Fe/I})_{\text{cell}}$ is the ratio of ^{59}Fe to ^{125}I radioactivity associated with the cell after t_0 min of incubation at 37°C, and $R(\text{Fe/I})_{\text{Tf}}$ is the ratio of ^{59}Fe to ^{125}I radioactivity in the iron-saturated ^{59}Fe, ^{125}I-labeled transferrin. The calculation is based on the observation that after 5–10 minutes of incubation with ^{59}Fe, ^{125}I-labeled transferrin, the cells reach steady-state levels of bound transferrin (^{125}I radioactivity) while a linear incorporation of ^{59}Fe into reticulocytes continues for at least 60 min (Hemmaplardh, Kailis & Morgan, 1974). The increase of the ratio $^{59}Fe/^{125}I$ radioactivity in the cell will be directly related to the number of iron-transferrin internalization cycles that have occurred during the incubation time.

^{59}Fe-Transferrin Binding

The rate of ^{59}Fe-transferrin binding at 37°C to surface receptors was determined in cells in which the receptor cycling process was inhibited by preincubation for 10 min at 37°C with 1 mM N-ethylmaleimide (Iacopetta & Morgan, 1983). The reaction was started with the addition of ^{59}Fe, ^{125}I-transferrin to final concentrations of 0.002, 0.01, 0.1, or 1.5 μM. At different times of incubation aliquots were centrifuged through dibutylphtalate and incubated for 60 min with 200 μl of rabbit plasma, centrifuged, and radioactivity determined in the supernatant. Controls of 4°C gave the same maximal amount of surface-bound transferrin and the same affinity constants indicating NEM does not affect transferrin binding.

Internalization of ^{59}Fe, ^{125}I-Transferrin

The rate of internalization of ^{59}Fe-transferrin was determined by incubating at 37°C, 400 μl of prewarmed cells with 800 μl of prewarmed ^{59}Fe, ^{125}I-labeled transferrin in Dulbecco's-BSA to give a final concentration of 1.5 μM in transferrin. At intervals from 10 to 180 sec, 120-μl aliquots of the incubation mixture were diluted into 1 ml of ice-cold Dulbecco's-BSA and immediately centrifuged for 10 sec in a microcentrifuge. To eliminate transferrin bound to external receptors, the cell pellet was resuspended in cold iron-saturated rabbit plasma as described above.

Dissociation of ^{59}Fe from ^{59}Fe, ^{125}I-Transferrin

Forty-μl of cells, prewarmed for 1 min at 37°C, were pulsed for 10 sec with 40 μl of prewarmed ^{59}Fe, ^{125}I-labeled transferrin (final concentration 1.5 μM) in Dulbecco's-BSA. The pulse was followed by a chase with 300 μl of prewarmed rabbit plasma which was 75% saturated with iron. The chase was stopped at different times by centrifugation of the mixture through a two-phase system consisting of 0.5 ml of dibutylphtalate layered on top of 0.3 ml of 25% sucrose which was 5 mg/ml in Nonidet P-40. The sucrose-Nonidet phase, which solubilized the cells, was made up to 3 ml with cold deionized water and centrifuged at 17,300 × g for 45 min. The procedure minimizes continued processing of internalized transferrin after a chase is terminated. Transferrin in the supernatant, which regularly corresponded to greater than 90% of the cell-associated transferrin, was then quantitatively absorbed to Sepharose-bound goat anti-rabbit transferrin antibodies (Glass et al., 1980). An estimate of the mean transferrin saturation at any time of chase was obtained by dividing the ^{59}Fe/^{125}I radioactivity ratio of the transferrin absorbed to anti-transferrin by the ^{59}Fe/^{125}I radioactivity ratio of the original, iron-saturated, ^{59}Fe, ^{125}I-labeled transferrin.

Membrane Non-Transferrin ^{59}Fe and ^{59}Fe Translocation into the Cytoplasm

The time needed for ^{59}Fe to translocate from the inside of the endocytic vesicle into the cytoplasm was determined using the following procedures. Cells were pulse chased as above and at different times of the chase the incubation mixture was centrifuged through a two-phase system consisting of 0.5 ml of dibutylphtalate layered on top of 0.3 ml of 25% sucrose. The cell pellet was lysed with 0.8 ml of 50 mM MES-NaOH buffer (pH 6.5), layered on top of 2.2 ml of 30% isotonic Percoll and centrifuged in a Sorvall refrigerated centrifuge for 60,000 × g for 1 min. The formed gradient was fractionated into (*i*) cytosol, corresponding to the upper 0.8 ml of the gradient; (*ii*) membrane and vesicle fraction, corresponding to the next 2 ml; and (*iii*) mitochondria, corresponding to the lower 0.2 ml of the gradient including the pellet. The amount of ^{59}Fe was determined as the sum of the radioactivity found in the cytosol and the mitochondrial fractions. Usually some ^{125}I-transferrin radioactivity corresponding to 5–15% of the cell-associated ^{125}I-transferrin was found in the cytosol fraction. The ^{59}Fe radioactivity corresponding to this transferrin, calculated from the measurement of ^{59}Fe dissociation at the same time of chase, was subtracted from the cytosol ^{59}Fe radioactivity, and added to the membrane ^{59}Fe fraction. The non-transferrin ^{59}Fe radioactivity in the membrane fraction from the Percoll gradient was determined by subtracting from the overall membrane ^{59}Fe radioactivity that radioactivity associated with transferrin at the same time of chase as determined from the ^{59}Fe dissociation measurements (Nunez & Glass, 1983).

Estimation of Rate Constants

Rate constants for iron dissociation from transferrin and the subsequent reactions were estimated using two different methods. In Method I rate constants were computed using the ENZFITTER nonlinear regression analysis program (Elsevier Biosoft) for first-order two-step reversible as well as two- and three-step irreversible reaction mechanisms (Chien, 1948; Frost & Pearson, 1953). In Method II rate constants were computed with a nonlinear regression analysis program kindly provided by Dr. Osvaldo Latorre (University of Chile) for irreversible flow between three compartments as described (Shepard, 1962), and using delay functions as described by Gex-Fabry and DeLisi (1984) for receptor-mediated endocytosis (Bridges et al., 1982; Hartford et al., 1982). In general, all models considered adequately described the data for ^{59}Fe dissociation from transferrin, and ^{59}Fe mobilization into the cytosol. However, data for nontransferrin membrane-associated ^{59}Fe could not be described by the two-step models using rate constants calculated from dissociation and mobilization data. Therefore, data for nontransferrin, membrane-associated ^{59}Fe was analyzed independently, and the resulting rate constants were tested for adequate descriptions of the dissociation and mobilization data.

Results

Transferrin Cell Cycle Time and Transferrin Receptor Distribution

The transferrin cell cycle time represents the average time for transferrin and its receptor to carry out an endocytic cycle for delivery of iron to the cell. The transferrin cell cycle time in rabbit reticulocytes was 255 ± 43 sec (mean ± SD of six experiments, range 220 to 324 sec). Total cell transferrin receptors averaged 132,000 ± 21,000 receptors per reticulocyte with 51,400 receptors, or 39 ± 5.7% of the total, exposed on the cell surface.

Rate of Fe-Transferrin Binding

By inhibiting internalization with NEM it was possible to measure the rate of binding of transferrin to the receptors on the surface of the reticulocytes. The apparent rates, as predicted (Schwartz, Fridovich & Lodish, 1982), were dependent on the concentration of transferrin and increased from 0.005 sec^{-1} at 1 × 10^{-9} M transferrin to 0.030 sec^{-1} at 1 × 10^{-8} M transferrin, and to 0.107 sec^{-1} at 1 × 10^{-7} M. The

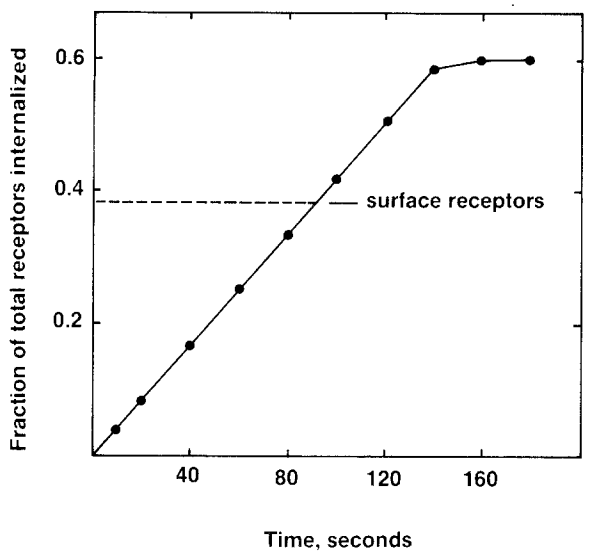

Fig. 1. Internalization of the iron-transferrin-receptor complex. Cells were incubated with ^{59}Fe, ^{125}I-labeled transferrin and internalized transferrin determined as described in Materials and Methods. Plotted is the fraction of total cell receptors internalized as a function of time of incubation. Results are the means of six independent experiments

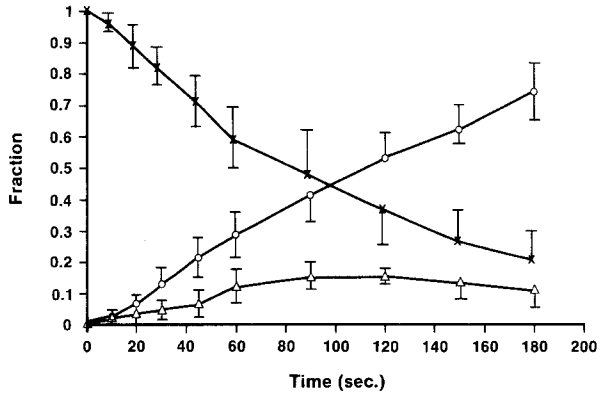

Fig. 2. Dissociation of iron from transferrin, vesicle membrane nontransferrin iron, and translocation of the internalized iron. Dissociation of iron from internalized transferrin was determined as described in Materials and Methods. Plotted as a function of the chase time are the fraction of: (*i*) the internalized iron bound to transferrin (×), (*ii*) the nontransferrin iron present in the membrane-vesicle fraction (△), and (*iii*) the iron present in the cytosolic plus mitochondrial fractions (○). Results are the means of six independent experiments

rate constant, k_1, was estimated to be 0.301 ± 0.042 sec^{-1} at 1.5 μM transferrin or 2.0 ± 0.3 × 10^5 M^{-1} sec^{-1} (mean ± SD of five experiments), a concentration of transferrin which will saturate the transferrin receptors (Iacopetta & Morgan, 1983) and the concentration at which all the subsequent ^{59}Fe uptake studies were performed.

Internalization of Iron-Transferrin-Receptor Complex

The internalization of the Fe-transferrin-receptor complex as a function of time is shown in Fig. 1. The rate of internalization averaged 520 ± 100 receptors per sec (mean ± SD of six experiments). The rate of receptor internalization remained constant after the receptors initially located on the cell surface were internalized (Fig. 1, dashed line). From the total number of receptors and the rate of receptor internalization an estimate of the transferrin cell cycle time can be obtained (Schwartz et al., 1982) independent of the cycle time as measured above. The internalization (and recycling) of a full complement of cell receptors takes about 250 sec, in good agreement with previous observations (Nunez & Glass, 1983). The $t_{1/2}$ for internalization of the surface receptors was 49 sec. Assuming pseudo-first-order kinetics, the average rate constant, k_2, for internalization of receptors located on the cell surface was 0.0140 sec^{-1}.

Dissociation of Iron from Internalized Transferrin

After internalization, iron dissociates from transferrin in a process requiring the acid milieu of the endocytic vesicle (Ciechanover et al., 1983; Iacopetta & Morgan, 1983; Nunez & Glass, 1985; Glass & Nunez, 1986). Figure 2 shows the fraction of the internalized iron bound to transferrin as a function of the time of chase. A 14.8 ± 1.9 sec processing time (mean ± SD of six experiments) can be estimated to occur before any iron dissociates from transferrin. The processing time for the first iron to dissociate was calculated from the pulse length (10 sec) plus the lag obtained from extrapolation of the iron dissociation curve to full transferrin saturation considering that the rate of dissociation was linear for the initial 60 sec of chase. Presumably the processing time reflects the time necessary to achieve acidification of the vesicle.

The Passage of Iron through the Vesicle Membrane Component(s)

The sum of the ^{59}Fe remaining on transferrin (Fig. 2) and ^{59}Fe in the cytoplasm (Fig. 2) does not equal all of the ^{59}Fe internalized during the pulse period. The difference, representing ^{59}Fe bound neither to transferrin nor yet in the cytoplasm, can be determined from the Percoll gradients to be in vesicle membranes. This compartment, shown in Fig. 2, represents at any time a relatively small, but easily

Table 1. Kinetic parameters for the various processes of iron uptake

Reaction	Rate constants (sec^{-1})	
	Method I	Method II
Iron dissociation from transferrin	0.0075 ± 0.0017	(17.1 sec) 0.009 ± 0.002
Membrane iron processing	0.0112 ± 0.0025	(34.4 sec)
Iron mobilization into cytosol	0.0343 ± 0.0118	0.0376 ± 0.0015

Shown are the rate constants for the various processes of iron uptake occurring after internalization of ferri-transferrin as estimated by the two methods described in Materials and Methods utilizing the data from Fig. 2. The values in parentheses refer to the delay functions as described by Gex-Fabry and DeLisi (1984) and are necessary in using Method II in order to obtain a fit to the experimental data.

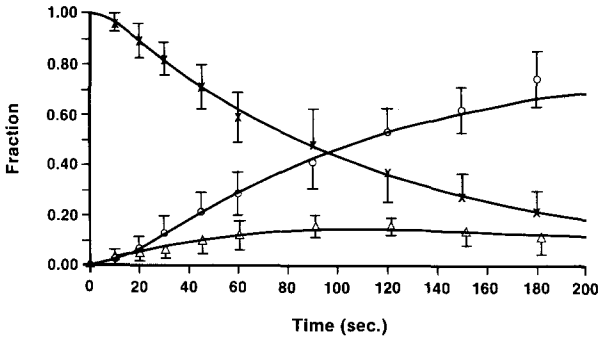

Fig. 3. Analysis of a three-step irreversible reaction to iron in the various intracellular compartments. As described in Materials and Methods rate constants for the solution to a series of first-order, three-step irreversible reactions were computed by nonlinear regression analysis utilizing from Fig. 2 the data for ^{59}Fe in the membrane and then applying the computed rate constants to ^{59}Fe in the other compartments. Shown are the curves generated by the regression analysis for the experimental data ± SD for iron dissociation from transferrin (×), iron in the membrane (△), and iron translocated into the cytosol (○)

detectable, fraction of the total iron. The iron content of the compartment increased with time up to 100 sec after the pulse and then decreased, behavior expected for a compartment kinetically placed between iron which had dissociated from transferrin and cytoplasmic iron.

Iron Mobilization into the Cytoplasm

The process of iron translocation into the cell cytoplasm is shown in Fig. 2. The shape of the curve is nearly the inverse of the time course of dissociation seen in Fig. 2, including the initial delay arising from iron dissociation and translocation followed by an increase of cytoplasmic iron.

Determination of Rate Constants for the Various Processes of Iron Uptake

The rate constants for the various steps in the iron uptake process are tabulated in the Table. As noted above the rate constants for Fe_2(III)Tf binding to the transferrin receptors and for the internalization of the transferrin-receptor complex can be calculated directly from the data in Figs. 1 and 2. The rate constants for the subsequent steps were computed by Method I using first-order two-step reversible, and two- and three-step irreversible, and by Method II using multistep irreversible flow mechanisms (Shepard, 1962; McCracken & Dorn, 1966; Schwartz et al., 1982; Gex-Fabry & DeLisi, 1984). As shown

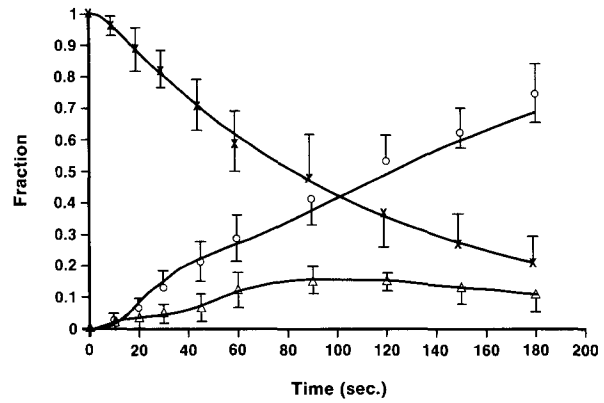

Fig. 4. The fit of irreversible flow and delay functions to iron in the various intracellular compartments. As described in Materials and Methods rate constants and delay functions were obtained using the methods described by Shepard (1962) and Gex-Fabry and DeLisi (1984). The delay function and rate constants computed for iron dissociation from transferrin were then used to calculate the delay function for iron processing in the membrane, and this delay function was then used to compute the rate constant for iron appearing in the cytosol. Shown are the fits obtained by nonlinear regression to the experimental data ± SD for iron dissociation from transferrin (×), iron in the membrane (△), and iron translocated into the cytosol (○)

in Figs. 3 and 4, both the three-step irreversible reactions and multistep irreversible flow mechanisms provide adequate descriptions of the data. Although dissociation and mobilization can be described by a two-step irreversible mechanism, membrane iron data cannot be described by this mechanism using the computed rate constants for

dissociation and mobilization (*data not shown*). Similarly, a two-step reversible reaction can describe membrane iron but the computed rate constants do not fit either dissociation or mobilization data. Overall, the results indicate at least one additional reaction occurs after iron dissociation from transferrin and before iron mobilization into the cytoplasm.

Discussion

Based on the measurement of iron passage through three compartments in reticulocytes — iron on internalized transferrin, non-transferrin membrane iron, and iron in the cytosol — models are presented for determining apparent rate constants of various steps involved in the intracellular handling of iron. Although, the kinetics of ferri-transferrin internalization and of the release of apo-transferrin have been measured (Iacopetta & Morgan, 1983; Woodworth et al., 1982; Klausner et al., 1983; Hanover et al., 1984), the rate constants for iron dissociation from transferrin, for processes involving nontransferrin iron associated with the membrane, and mobilization of iron from the membrane into the cytosol have not previously been defined. Further, these measurements establish the sequence of events in the transport of iron after the initial step of ferri-transferrin internalization and establish testable models for the handling of iron.

It is apparent from the data in Figs. 1 and 2 that internalized iron proceeds through a minimum of three compartments: iron on transferrin, iron in the membrane, and iron in the cytosol. Our present studies used methodology to immediately centrifuge cells into a lysis solution to prevent continued processing of Fe(III)Tf and as such demonstrated a longer processing time before iron could be removed from transferrin than previously found (Nunez & Glass, 1983). The processing could reflect either the time needed to fully acidify the endocytic vesicle or some other reaction such as removal of clathrin from coated vesicles which might, by an as yet undescribed mechanism, be required for removal of iron from transferrin.

Analysis of the data using the irreversible flow model (Method II) requires the calculation of processing or delay times (Gex-Fabry & DeLisi, 1984) in order to generate the fits shown in Fig. 4. Similar delays have been noted in other uptake processes involving endocytosis (Brown, Yeh & Holley, 1979; Bridges et al., 1982; Harford et al., 1982; Wiley & Cunningham, 1982; Gex-Fabry & DeLisi, 1984). The calculated delay time for reactions preceding ^{59}Fe dissociation from transferrin was 17.1 sec, from the beginning of the 10-sec pulse, compared to the experimentally determined 14.8-sec lag before dissociation was observed. Assuming that a single process is described by the 17-sec delay time and that the process has progressed to 90% completion during 17 sec (i.e., $t_{1/2}$ = 4.25 sec), then the reactions preceding dissociation are characterized by an apparent rate constant of about 0.16 sec^{-1}.

Analysis using a three-step irreversible reaction mechanism (Method I) was also adequate to describe the experimental data. Fitting the data for ^{59}Fe in the membrane fraction using a three-step irreversible reaction mechanism yielded rate constants for iron dissociation and mobilization that were similar to those obtained from the irreversible flow model (Table). As seen in Fig. 3, the three-step model adequately describes ^{59}Fe dissociation from transferrin including the apparent delay time without invoking vesicle processing. This result suggests a less complex model for events preceding iron dissociation in which any processing steps are either too rapid to be detected by these experiments or are not required for iron dissociation from transferrin. The rate constant for endocytic processing estimated from the delay time (0.16 sec^{-1}) is much greater than the other computed parameters and lends support to a mechanism which includes rapid endocytic processing. These results indicate that endosome processing is not rate determining in this experimental system and that reactions separate from transferrin endocytosis are kinetically important.

The presence of additional reactions during the transient sojourn of ^{59}Fe in the membrane is suggested by the inability of two-step reversible and irreversible reactions to adequately describe the data for ^{59}Fe in the membrane (*data not shown*). In addition, both three-step irreversible and multistep irreversible flow models require at least one reaction to occur while iron is associated with the membrane. Hence, it seems reasonable that another reaction occurs while the iron is in the membrane compartment. However, significant differences between apparent rate constants computed by the three-step irreversible reaction scheme and the multistep irreversible flow model are observed. As shown in the Table, the irreversible flow model requires a delay time of 34.4 sec to achieve the fit shown in Fig. 4 or, again assuming about 90% completion of the process ($t_{1/2}$ = 8.6 sec), a rate constant of about 0.081 sec^{-1}. In contrast, the three-step irreversible reaction scheme gives a rate constant of 0.0112 sec^{-1} for the presumed membrane processes. Although the origins of these differences are not entirely clear, it is likely that some of these discrepancies arise from the numerous approximations inherent in the irreversible flow model and the stationary state assumed in deriving the solution of the three-step irreversible

reaction scheme (Chien, 1948; Frost & Pearson, 1953; Gex-Fabry & DeLisi, 1984)

Two possibilities for reactions involving membrane iron are either the reduction of iron or the translocation of iron from the *cis* or intralumenal surface of the endocytic vesicle membrane surface to the *trans* or cytoplasmic surface. An erythrocyte plasma membrane oxidoreductase has been described (Zamudio, Cellino & Canessa-Fischer, 1969), evidence for the reduction of iron after release from transferrin has been presented in reticulocytes (Nunez, Cole & Glass, 1983*b*), and in other tissues the role of a NADH-reductase has been implicated in iron uptake (Sun et al., 1987*b*; Thorstensen, 1988). The role for translocation of iron across the membrane is derived from the finding that transferrin resides within the endocytic vesicle (Choe et al., 1987) mandating that the dissociated iron requires either a channel or carrier to cross to the cytosol (Egyed, 1988; Morgan, 1988; Sturrock, et al., 1990). Therefore, it is likely that both reactions are involved in membrane iron processing.

Based on these results and mechanistic considerations a minimum sequence of reactions involved in iron uptake can be proposed:

Binding:

(1) $Fe_2(III)\text{-Tf} + R \rightarrow [Fe_2(III)\text{-Tf-}R]_{out}$

Internalization:

(2) $[Fe_2(III)\text{-Tf-}R]_{out} \rightarrow [Fe_2(III)\text{-Tf-}R]_{in}$

Processing:

(3) $[Fe_2(III)\text{-Tf-}R]_{in} \rightarrow [Fe_2(III)\text{-Tf-}R]^*_{in}$

Acidification:

(4) $[Fe_2(III)\text{-Tf-}R]_{in} + 4H^+ \rightarrow [\text{Tf-}R\cdots(Fe_2(III)]_{in}$

Dissociation:

(5) $[\text{Tf-}R\cdots Fe_2(III)]_{in} + IBP \rightarrow [R\text{-Tf}] + Fe(III)\text{-IBP} + HCO_3$

Reduction:

(6) $Fe_2(III)\text{-IBP}_{cis} + D^- \rightarrow Fe_2(II)\text{-IBP}_{cis} + D^+$

Translocation:

(7) $Fe_2(II)\text{-IBP}_{cis} \rightarrow Fe_2(II)\text{-IBP}_{trans}$

Mobilizatin:

(8) $Fe_2(II)\text{-IBP}_{trans} + 2C \rightarrow 2Fe(II)\text{-}C + IBP$

The initial reactions of binding (Reaction 1) and internalization (Reaction 2) have been meticulously discussed (Klausner et al., 1985) and are far more complex than depicted in the reaction scheme. Four steps have been added to the scheme presented in the Introduction: processing (Reaction 3) and acidification (Reaction 4) are proposed to occur after transferrin internalization, while reduction (Reaction 6) and translocation (Reaction 7) occur after dissociation. To determine which of these reactions are indeed required and the sequence of the reactions, an in vitro model has been developed (Nunez et al., 1990) in which endocytic vesicles are isolated with iron still on transferrin and in which the acidification process can be initiated. In preliminary studies with this in vitro system, vesicle acidification followed by reduction of iron by a NADH-reductase appears to be required for translocation of iron across the endocytic membrane.

It should be noted that the proposed sequence of events contrasts to that reported for hepatocytes (Sun et al., 1987*b*; Thorstensen, 1988) where reduction of iron is also essential for iron transport but where the reductive process occurs at the cell surface. Nevertheless, in these studies as well as others (Egyed, 1988; Morgan, 1988; Sturrock, et al., 1990) iron has been demonstrated to cross the plasma membrane. Acidification of the microenvironment at the hepatocyte surface may be required as well but may be mediated through a Na^+/H^+ exchanger (Sun et al., 1987*a*). The processes in these systems are similar in that iron crosses the membrane to gain access to the cytosol.

Although the intent of the study was to concentrate on the intracellular passage of iron, the observations on the handling of ferri-transferrin were interesting. As may be inferred from previous work (Glass & Nunez, 1986) the rate of internalization of the $Fe_2(III)$Tf-*R* complex was constant even after all the receptors initially exposed on the cell exterior had been internalized. This result can be explained by a model in which internalization and externalization of the receptors are coupled and in steady state; that is, for a receptor to emerge to the cell surface another must be internalized. This model also demands that a newly emerged receptor have a finite residence time on the cell surface. These predictions are consistent with the observation that the rates of transferrin internalization and externalization are equal in rabbit reticulocytes (Iacopetta & Morgan, 1983), and the observation that endocytosis of transferrin in K562 (Watts, 1985) and HeLa (Ajioka & Kaplan, 1986) cells is a constitutive process in which the transferrin receptor may continue to be endocytosed in the absence of ligand. It is possible that in other cell types a different mechanism may apply, as with human fibroblasts in which a lag in the internalization of transferrin has been described (Wiley & Kaplan, 1984).

The reactions involved in iron transport across endocytic membranes may be similar for cellular systems that have been studied regardless of differences in transferring cell cycle times (Ciechanover, et al., 1983; Iacopetta & Morgan, 1983). Although these differences may be due to endocytic processing and/or internalization phenomena, physical and

chemical constraints are likely to substantially limit the number of ways iron can cross a membrane. It is apparent that processing of non-transferrin membrane-associated iron is of substantial kinetic significance. It is also likely that reactions involving membrane iron include iron reduction as well as translocation and that these reactions are independent of the intracellular processing of transferrin. However, several questions remain unanswered concerning the exact sequence and nature of intermolecular processes. For example, the importance of intermolecular complex formation and conformational changes associated with proton transfer, electron transfer, and/or iron translocation remain unexplored. In addition, factors that influence acidification by interactions with the H^+-ATPase have not been determined. Although, these phenomena are beyond the scope of this report, they are currently under investigation using an in vitro model of iron mobilization from isolated endosomes.

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