Iron Acquired from Transferrin by K562 Cells Is Delivered into a Cytosolic Pool of Chelatable Iron(II)*

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The release of iron from transferrin (Tf) in the acidic milieu of endosomes and its translocation into the cytosol are integral steps in the process of iron acquisition via receptor-mediated endocytosis (RME). The translocated metal is thought to enter a low molecular weight cytosolic pool, presumed to contain the form of iron which is apparently sensed by iron responsive proteins and is the direct target of iron chelators. The process of iron delivery into the cytoplasmic chelatable pool of K562 cells was studied in situ by continuous monitoring of the fluorescence of cells loaded with the metal-sensitive probe calcein. Upon exposure to Tf at 37 °C, intracellular fluorescence decayed, corresponding to an initial iron uptake of 40 nm/min. The Tf-mediated iron uptake was profoundly inhibited by weak bases, the protonophore monensin, energy depletion, or low temperatures (< 25 °C), all properties characteristic of RME.

Cell iron levels were affected by the slowly permeating chelator desferrioxamine only after prolonged incubations. Conversely, rapidly penetrating, lipophilic iron(II) and iron(III) chelators, precluding swift increases in cell calcein fluorescence, equivalent to sequestration of 0.2–0.5 μM cytosolic iron, depending on the degree of pre-exposure to Tf. Addition of iron(III) chelators to permeabilized 2,2'-bipyridyl-treated cells, failed to reveal significant levels of chelatable iron(III). The finding that the bulk of the intracellular pool is comprised of iron(II) was corroborated by pulsing K562 cells with Tf-59Fe, followed by addition of iron(II) and/or iron(III) chelators and extraction of chelator-59Fe complexes into organic solvent. Virtually all of the accumulated 59Fe in the chelatable pool could be complexed by iron(II) chelators. The cytosolic concentration of iron(II) fluctuated between 0.3 and 0.5 μM, and its mean transit time through the cytoplasmic pool was 1–2 h. We conclude that after iron is translocated from the endosomes, it is maintained in the cytosol as a transit pool of chelatable iron(II). The osnitive absence of chelatable iron(III) implicates the intracellular operation of vigorous reductive mechanisms.

Cell iron metabolism is characterized by regulated trafficking of the metal between different cellular stations. In animal cells, these include supply stations such as transferrin (Tf)1.

* This work was supported in part by the Israel National Science Foundation and National Institutes of Health Grant AI20342. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Tf, transferrin; RME, receptor-mediated endocytosis; BIP, 2,2'-bipyridyl; PDT, 3-(2-pyridyl)-5,6-diphenyl-

containing endosomes and cytosolic ferritin and target stations in the cytosol and organelles (3). The crossroad between the different stations has been associated with a cytosolic pool of low molecular weight iron (2–4) which is also presumed to be the immediate intracellular target of iron chelators (5, 6). The trafficking of iron is assumed to be regulated by appropriate adjustments in the levels of membrane Tf receptors (Tf-R) (iron acquisition) and cytosolic ferritin (iron storage). A key step in that regulatory process is the monitoring of a loosely bound form of the iron in the "chelatable pool," which reflects the metabolic status of iron in the cell. This has been attributed to a cytosolic iron-responsive protein which contains a 3Fe-4S cubane cluster that reversibly forms a 4Fe-4S complex in the presence of excess iron. Partial iron occupancy of the cluster has been implicated in the conversion to the iron-responsive protein conformation which is active in translational regulation of cellular Tf-R and ferritin levels (7).

The importance of the chelatable iron pool in iron metabolism is generally accepted, although it has been difficult to assess both quantitatively and qualitatively. Analytical techniques which depend on cell-disruptive steps are of limited use, due to the dynamic nature of the cytosolic iron pool and the metastable properties of the metal in ambient conditions. Nonetheless, cell extracts were shown to contain a chelatable form of iron which is distinct from tightly bound metal (6, 8–10). Likewise, soluble cell fractions have been found to contain low molecular weight forms of iron which are chromatographically separable from ferritin and other iron-binding proteins (2–4). A number of putative iron ligands have been identified, including peptides (11), proteins (12, 13), and nucleotides (4). However, their association with the chelatable iron pools prevailing in intact cells has not been unequivocally established. The same degree of uncertainty exists regarding the relative levels of di- and trivalent iron forms present in the cytosolic (chelatable) pool. Physicochemical determinations of iron in tissue and cell preparations implicate iron(II) as the most abundant low molecular weight form of the metal (25). However, chemical determinations of iron in cell and tissue extracts have given variable values, a situation which reflects the propensity of iron(II) for oxidation (2, 4, 6, 10).

In this work we assessed the in situ levels and chemical forms of chelatable iron which prevail in the cytosol of K562 cells. We used the method of continuous monitoring of intracellular fluorescence associated with the metal-sensitive probe calcein (1). This method, in conjunction with the application of permeant and impermeant iron(II) and iron(III) chelators, pro-
vided information regarding: (a) the dynamics of cytosolic (chelatable) iron delivery from endocytosed Tf, (b) the chemical form (i.e. valence) of iron associated with the chelatable pool, and (c) the maintenance of that pool under different iron supply conditions. A complementary methodology was adapted here for quantitative analysis of cell chelatable iron, based on labeling with radioactive $^{59}$Fe and direct cell extraction of chelator-$^{59}$Fe complexes with an organic solvent. It enabled us to reinforce the basic findings obtained by non-invasive fluorometry and also to provide estimates for the mean transit time of Tf-derived iron through the chelatable pool. The methodologies offered also a convenient means for assessing chelators’ capacity for iron sequestration and removal from cells, with implications for pathological conditions of iron overload.

EXPERIMENTAL PROCEDURES

Materials—Calcine and its acetoxyethyl ester (calcine-AM) were obtained from Molecular Probes (Eugene, OR). Ionomophore A23187, octyl $\beta$-o-glucoside detergent, ferrous ammonium sulfate (FAS), and diethyl-\textit{enriaminepentaacetic acid (DTPA), nitridotriacetic acid (NTA), and bathophenanthroline disulfonic acid (BPS) were from Sigma. All materials for the biotinylated Tf were obtained from Becton-Dickinson (Rockville, MD). Silica-aldehyde and pyridoxal 5'-azothiazinyl hydrzones (SH and PIH) were generous gifts from Dr. P. Ponka (Lady Davis Institute for Medical Research, Montreal, Canada); methylanthranillic DFO (MAD) was prepared by Dr. Brenda Meister (Weizmann Institute, Rehovot, Israel). Chromatographically pure Tf and apoTf were from Kama Da Industries (Kibbutz Kama, Israel).

Cell Treatments and Loading with Calcein—Human erythroleukemia K562 cells were propagated in $\alpha$-MEM medium containing 7% fetal calf serum supplemented with $\gamma$-glutamline and antibiotics (1). Cells were used at a density of 2-3 x 10$^6$ cells/ml with 0.125 $\mu$g of calcine-AM for 5 min at 37°C in HEPES-buffered, bicarbonate-free $\alpha$-MEM medium containing 20 $\mu$g HEPES, pH 7.3 ($\alpha$-MEM-HEP), washed with $\alpha$-MEM-HEP medium, and maintained at room temperature until use. Just prior to measurements, 1 ml of calcine-loaded cell suspension (approximately 5 x 10$^6$ cells) was centrifuged in a microcentrifuge, and the cells were resuspended in 2 ml of prewarmed 150 $\mu$g NaCl, 10 $\mu$g HEPES-Tris, pH 7.3 (HBS buffer).

Fluorescence Measurements and Calibration—Calcine fluorescence (excitation, 486 nm; emission, 517 nm; slit, 10 nm) was measured in a Perkin-Elmer LS-5B fluorimeter equipped with a temperature-controlled cuvette holder and magnetic stirrer. Data were recorded on line on a GP-100 Graphics Printer (Perkin-Elmer). Optimal fluorescence setting might differ for different instrument brands.

The concentration of free calcine inside cells was found to be directly proportional to the length of incubation and calcine-AM concentration. It was estimated as follows: approximately 2 x 10$^6$ calcine-loaded cells were centrifuged in a narrow-bore microcentrifuge giving a packed cell volume of 20 $\mu$l. The cell pellet was solubilized in 2 ml of HBS buffer containing 1.5% octyl glucoside and 200 $\mu$g DTPA. The calcine concentration in the cell lysate was determined from its fluorescence and compared with that of calcine standards in the same solution, giving an intracellular calcine concentration of 20 $\mu$g.

The relationship between fluorescence changes and intracellular iron concentration was determined for each experiment as follows. A baseline signal was obtained for a suspension of cells in the cuvette. Ionomophore A23187 (10 $\mu$g/well) was added (this produced a < 5% change in the signal), followed by serial doses of 0.5 or 1.0 $\mu$g iron(II) added as FAS, and the change in the fluorescence corresponding to each dose was recorded.

Preparation of Tf-$^{59}$Fe—The procedure of Klausner et al. (14) with slight modifications was used. $^{59}$FeCl$_3$, 0.4 mCi (Amersham, Amersham, Bucks, UK) was complexed to NTA at a ratio of 0.18 $\mu$mol of Fe:1.8 $\mu$mol of NTA, followed by the addition of 0.05 $\mu$mol of apotransferrin dissolved in 0.5 $\mu$mol Tris-HCI, 0.1 $\mu$mol NaHCO$_3$, pH 8.5. $^{59}$Fe was separated from non-bound $^{57}$Fe by gel filtration on a PD-10 column pre-equilibrated with HBS (Pharmacia, Uppsala, Sweden).

Quantitative Extraction of Chelator-$^{59}$Fe Complexes—A $^{57}$FeCl$_3$NTA complex at a 1:20 molar ratio was prepared and diluted to a final concentration of 0.43 $\mu$mol $^{57}$Fe and 0.088 $\mu$mol NTA. Various chelators were added to the cell suspensions, followed by incubation with radioactive $^{57}$Fe and direct cell extraction of chelator-$^{57}$Fe complexes (20). The temperature of the cell suspension in the cuvette was maintained at 37, 25, and 20°C, as indicated at the beginning of each trace. In each case a base-line signal was established for 4 min prior to the addition of 50 $\mu$mol iron-saturated transferrin (Tf, first arrow). After 10-12 min, 10 $\mu$mol ferrous ammonium sulfate (FAS, second arrow) was added in each case. The correlation between the changes in fluorescence and intracellular iron concentration (scale shown at bottom left) was determined as described under “Experimental Procedures.”

RESULTS

TF-mediated Uptake of Iron and Its Inhibition by Blockers of Receptor-mediated Endocytosis—We have utilized the fluorescent probe calcine to study the uptake of iron from Tf. Addition of 50 $\mu$mol Tf to a suspension of calcine-loaded K562 cells at 37°C resulted in a slow time-dependent quenching of intracellular fluorescence (Fig. 1). The quenching rate was linear for 8-12 min, after which it leveled off. The intracellular location of calcine was ascertained in this experiment by the presence of specific anti-calcine antibody which quenches all extracellular calcine fluorescence; intracellular calcine is not accessible to
the antibody. As shown in Fig. 1, the estimated uptake of iron derived from Tf was 0.04 μM iron/min at 37 °C and was virtually undetectable at temperatures below 25 °C (bottom traces in Fig. 1). In contrast, the uptake of iron(II) from the medium (10 μM FAS) occurred at 0.16 and 0.14 μM Fe/min at 37 and 25 °C, respectively. It was also less sensitive to temperature, as expected for a facilitated diffusion-controlled process (see also Ref. 1). In order to confirm that the quenching of intracellular calcein is the consequence of the established endocytic pathway of uptake of iron from Tf, we tested the effects of several classical inhibitors of RME (14, 15). Monensin, chloroquine, and NH₄Cl, all of which alkalinize acidic compartments, and the metabolic poison sodium azide, inhibited the rate of quenching of calcein (Table I).

Detection of Chelatable Intracellular Iron with Calcein—The affinity constants of calcein for iron(II) and (III) are very similar to those of EDTA (10¹⁴ and 10²⁴ M⁻¹), respectively, based on our measurements of the capacity of EDTA to prevent the quenching of calcein fluorescence by iron(II) and (III). These estimates are compatible with the structure of calcein, since it contains two ethyleneaminediacetate iron binding moieties identical to those in EDTA. Addition of iron(II) as FAS to a calcein solution at a 1:1 molar ratio resulted in 41% quenching of the fluorescence (Fig. 2, trace a). This quenching was readily reversible by the addition of iron(III) chelators such as SIH and DFO (Fig. 2, traces c and d), but not by iron(II) chelators such as BIP, BPS, or PHE (Fig. 2, trace e). The latter group caused quenching only in the presence of ascorbate, as exemplified for BIP (Fig. 2, traces a and b).

The degree of quenching of cytoplasmic calcein by a high affinity iron chelator could provide an indication of the amount of cellular iron bound to calcein (Fig. 3). Exposure of calcein-loaded K562 cells to the chelators DFO and BPS caused no short term change in the signal, probably due to their membrane impermeability. However, the permeant chelator BIP (but not its inactive isomer 4,4′-bipyridyl, not shown), produced a marked increase in the fluorescence, corresponding to 0.27 μM iron (Fig. 3, trace a). Following exposure of the cells to 50 μg/ml Tf for 6 min, the magnitude of BIP-chelatable iron level increased to a value of 0.51 μM (Fig. 3, trace b). This increment is in full agreement with the rate of iron uptake of 0.04 μM/min estimated from Fig. 1 and indicates that BIP chelates the accumulated calcein-bound iron with high efficiency. Exposure of the cells to 10 μM iron(II), in the form of FAS, produced a further rise in the BIP-chelatable iron level (0.76 μM) (Fig. 3, trace c). However, when high levels of iron(II) were acquired from FAS, a portion of the internalized iron was lost from calcein after removal of the external FAS. This is manifested by the slow upward trend of the fluorescent signal in trace c after washing of the cells. Whether this occurred due to efflux of iron out of the cells down the outwardly directed concentration gradient, or removal of iron from calcein by cytoplasmic-binding factors, is not clear.

Demonstration That the Chelatable Iron Pool Detected with
Calcein Consists of Iron(II)—The purpose of the experiments shown in Fig. 4 was to assess (i) whether all the calcein-detectable cytoplasmic iron is iron(II) (i.e., BIP-reactive), (ii) the relative levels of iron(II) and iron(III) in the chelatable pool, and (iii) whether the presence of calcein affected Tf-mediated iron(II) accumulation. Calcein-preloaded cells showed detectable levels of BIP-reactive iron(II), which increased after incubation with Tf (Fig. 4A, preloaded). Permeabilization by addition of a non-ionic detergent (0.75% octyl β-D-glucoside) caused transient noise due to changes in light scattering (shown as an interruption in the I trace). Ascorbate was added to reduce any available iron(III) to iron(II) and to make it available for chelation by BIP. It led to a 15–20% increase in fluorescence. This indicates that the level of chelatable iron(III) present in the intact cytosol is relatively small and probably inaccessible to or unaffected by BIP. Moreover, even the above figure of 20% might be overestimated, since it might represent BIP-inaccessible iron(II) that had undergone rapid oxidation upon cell solubilization. DFO was added at the end of each set of experiments to ensure complete removal of all iron forms (II and III) bound to calcein. Similar results were obtained when the order of exposure was reversed, i.e., the cells were first exposed to Tf and then loaded with calcein (Fig. 4B, postloaded). Addition of BIP 3 min after the detergent was ineffective in restoring fluorescence, unless reduction was artificially imposed by 50 μM ascorbate (Fig. 4C).

Effect of DFO Treatment on the Chelatable Iron(II) Pool—In order to confirm that the calcein/BIP method provides a valid measure of the chelatable pool of cytoplasmic iron, K562 cells were pretreated with various concentrations of DFO for 20 h and then assayed for steady-state chelatable iron(II) as well as for delivery of iron(II) into this pool. As shown in Fig. 5 (left side traces), the steady-state calcein/BIP-detectable iron(II) concentration was 0.40, 0.18, 0.13, and 0.06 μM, respectively, in cells treated with 0, 50, 100, and 300 μM DFO. The delivery of iron from Tf into the calcein-detectable pool was also affected by DFO, as shown in Fig. 5 (right side traces). After exposure of the cells to 50 μg/ml of Tf for 8–10 min, the estimated chelatable iron(II) concentration (determined from BIP quenching) was 0.81, 0.76, 0.32, and 0 μM, respectively, in cells treated with 0, 50, 100, and 300 μM DFO. The addition of SIH (a membrane permeable chelator which binds both iron(II) and (III)) revealed the presence of a minor BIP-inaccessible pool, which may correspond to iron(III); however, this pool was not significantly affected by the DFO pretreatments.

Quantitation of Chelator-bound 55Fe(II) and (III) by Organic Solvent Extraction—We applied an independent method of estimating the chelatable iron(II) pool using 55Fe as a tracer. The water-soluble complex 55Fe(III)-NTA was 98% retained in the water phase in the absence of chelators, but in the presence of 100 μM PIH, SIH, and MAD, >97% of the 55Fe was found in the alcohol phase. This occurred regardless of the presence or absence of ascorbate, which is consistent with the known capacity of these chelators to bind both iron(II) and iron(III) (8, 18). In contrast, the iron(II)-specific chelators BIP and PHE translocated the 55Fe into the organic phase only in the presence of ascorbate. The slightly elevated chelation of 55Fe(II) by PHE can be attributed to a shift in the iron(II)/iron(III) equilibrium induced by this powerful iron(II) chelator. In the case of DFO, only 42% of the total DFO-55Fe(III) complex was extracted into benzyl alcohol, whereas 44% was retained in the water phase, and 14% was presumably trapped at the interface. This occurred because the partition of the chelator-iron complexes into benzyl alcohol is directly related to their hydrophobicity, so the hydrophilic DFO was extracted inefficiently. The DFO analogue, MAD, contains a methylanthranilate group attached to DFO, which renders the molecule sufficiently hydrophobic to be organic-solvent extractable.

The benzyl alcohol extraction system was applied to the...
determination of chelatable iron in intact cells (Fig. 7). K562 cells which had accumulated $^{55}$Fe from Tf were incubated for 10 min with 200 $\mu$M chelators, and the chelator-bound $^{55}$Fe associated with the cells after washing was determined in the cell extracts. In the absence of added chelators 1.0 $\pm$ 0.4% of the total cellular $^{55}$Fe was extracted into the organic phase, whereas 82 $\pm$ 4.6% was water-soluble. The residual 15–17% of the $^{55}$Fe was principally found in the denatured protein fraction which precipitated at the water-benzyl alcohol interphase. The iron(II)-specific chelators BIP, PHE, and PDT chelated 15–22% of the cellular $^{55}$Fe when they were added to intact cells. Similar values were obtained when these chelators were added to cells together with Triton X-100 detergent during preparation of the cell extracts (data not shown).

The iron(II)/(III) binding chelators PIH, SIH, and MAD chelated 14.5–16.5% of the $^{55}$Fe when they were added to the cells together with the solubilizing buffer (systems labeled with an asterisk in Fig. 7). However, in intact cells they appeared to chelate only 8–9% of the $^{55}$Fe. Examination of the cell supernatants following the 10-min incubation with the chelators revealed that although BIP and PDT did not cause a significant efflux of $^{55}$Fe from the cells, PHE, PIH, SIH, and MAD caused losses of $^{55}$Fe from the cells ranging from 6 to 30% (data not shown), presumably due to their capacity for shuttling iron in and out of cells (8). This would explain the apparently reduced chelation by PIH, SIH, and MAD in intact cells.

An average of 0.011 nmol of $^{55}$Fe/5 $\times$ 10$^6$ cells were incorporated from Tf during the 30-min incubation, of which an average of 0.0022 nmol of $^{55}$Fe/5 $\times$ 10$^6$ cells were present in the chelatable iron(II) pool. Using an approximate value of 1000 fl/cell for the cellular volume (19), we calculate that the iron(II) concentration in the chelatable pool is approximately 0.44 $\mu$M.

Transit Time Kinetics of the Chelatable Iron(II) Pool—The organic extraction system permitted the estimation of the time spent by incoming $^{55}$Fe in the chelatable pool (Fig. 8). At the conclusion of a 30-min pulse with Tf-$^{55}$Fe, 18.1% of the incorporated $^{55}$Fe was found in the BIP- and PIH-chelatable pool. This level decreased in a linear fashion for 2 h (down to 3.4%) and thereafter declined more slowly. The $^{55}$Fe in the non-chelatable pool increased proportionately. Therefore the estimated transit time through the chelatable pool is approximately 2 h. Since this value was obtained by chasing the incorporated $^{55}$Fe with saturating amounts of unlabeled Tf-iron, it is presumed to represent the maximal rate of flow of iron through the system.

DISCUSSION

Calcein as a Fluorescent Probe for Iron in Solution—Calcein is a derivative of fluorescein with two metal binding moieties and affinities for iron(II) and iron(III) identical to those of EDTA (10$^{14}$ and 10$^{24}$ M$^{-1}$, respectively, in aqueous salt solutions). Quenching of fluorescence ensues upon binding of iron and other metals, but not Ca(II) or Mg(II), even at 1000-fold excess. In physiological salt solutions, binding to iron(II) is considerably faster than to iron(III), reflecting the different solubility and chemical activity of the two forms of iron (1). However iron(II) rapidly oxidizes upon binding to the probe in ambient air, as noticed previously with other chelators (20). Consequently, the quenched fluorescence of the calcein-iron(II) complex in solution is fully restored by addition of excess
Chelatable iron(II) pool in K562 cells

Chelators such as DTPA, DFO, SH, and PIH, but not by iron-(II)-specific chelators such as BIP, PHE, PDT, or BPS. The latter require the presence of a reducing agent (Fig. 2).

Calcein as an in situ indicator of the chelatable iron(II) pool—Calcein loaded into cells was recently used by us for measuring uptake of non-transferrin bound iron(II) and other metals into K562 and other cells. Fluorescent cell imaging showed it to be homogeneously distributed in the cytosol (1). The sensitivity of iron uptake measurements was found to be inversely proportional to the intracellular calcein concentration, i.e. the lower the background fluorescence, the greater the iron-mediated quenching response. Hence, Tf-mediated iron uptake could be detected in K562 cells loaded with 0.125 M iron at a lower signal-to-noise ratio at lower calcein levels.

In this study, but not with 0.5 M iron, calcein showed it to be homogeneously distributed in the cytosol (1), but not with 0.5 M (1) calcein-AM, containing, respectively, 20 and 140 M intracellular calcein, presumably due to the higher signal-to-noise ratio at lower calcein levels.

The presence of 20 M calcein in the cytoplasm does not appear to significantly affect the chelatable iron pool, since (i) calcein-detectable cellular iron levels were similar when cells were loaded with calcein before or after exposure to Tf (Fig. 4, A and B), and (ii) in cells labeled with 55Fe and then loaded with increasing concentrations of calcein (20–140 M inside) and the level of Tf-chelatable 55Fe increased by 89% relative to controls (not shown). Also, in cells preloaded with calcein (20 M inside) and then labeled with Tf-55Fe, the level of the BIP-chelatable 55Fe pool was increased by 18% relative to controls (not shown), indicating that calcein competes weakly with intracellular ligands for iron(II) entering the cytoplasm.

The present assessment of the chelatable iron pool in situ in K562 cells is based on the availability of fast permeating high affinity chelators and their capacity to abstract iron from calcein, causing a dequenching of its fluorescence. Although only data with BIP are shown, virtually identical responses were obtained with two other iron(II) chelators, PHE and PDT, and with the iron(II) and (III) chelators, SIH and PIH. All these data are not shown.

These four chelators are known to be present in the cytoplasm of K562 cells (3). However, with SIH, DFO also failed to increase the fluorescence in solubilized cells pre-exposed to BIP (Fig. 4). Taken together, the data shown in Figs. 3–5 indicate that the calcein-detectable iron pool of K562 cells is composed primarily of iron(II) with a possible minor component of iron(III). This conclusion is consistent with our previous results that the divalent metal-specific ionophore A23187, in the presence of an extracellular metal sink, DTPA, could mobilize iron out of the cells (1). The present value of 0.2–0.5 M chelatable iron might be somewhat underestimated, as some chelatable iron might be bound to ligands with higher affinity than calcein. Nonetheless, the above value is comparable with that obtained in 55Fe labeling experiments (see below) and with rough estimates in the literature of 0.5–1.0 M cytosolic iron (3, 5).

Assessment of the Chelatable Iron(II) Pool by 55Fe Labeling—An independent confirmation for the idea that iron(II) is the major component of the chelatable iron pool was obtained with the aid of a method for hydrophobic metal-chelator extraction into benzyl alcohol (17), which allowed the separation of non-chelated iron from chelator-iron complexes (Fig. 6). This technique enables us to quantify the chelator-55Fe complexes in intact cells and in cell extracts (7). The estimated 55Fe-labeled chelatable pool was similar for both iron(II)-specific and iron(II) and iron(III) chelators from which we deduced that it is comprised primarily of iron(II). The value of a chelatable pool of 0.44 M, which was similar to the one estimated with calcein (0.2–0.5 M in Figs. 3–5), represented approximately 18% of the total 55Fe acquired iron Tf within 30 min. The bulk of this iron transited through the cytosol in about 2 h. Similar results were obtained in studies with rat hepatocytes labeled with 59Fe and analyzed for DFO-chelatable 59Fe (6).

A cellular iron(II) pool has been suspected previously (3, 24) and has been indicated by spectroscopic studies of rat liver extracts by electron paramagnetic resonance in conjunction with iron(II) chelation (10) and of hepatoma cells by 57Fe Mossbauer spectroscopy (25). However, these approaches entailed disruption of cells, possibly inducing changes in the iron distribution (3). Our spectroscopic approach allowed dynamic and in situ monitoring of the cytosolic chelatable iron pool in living cells without violation of their integrity and proliferation capacity.2 Once cell integrity was disrupted, the technique fully reflected iron(II) oxidation by ambient air, as shown by BIP’s failure to recover the metal-quenched fluorescence (Fig. 4C).

The present approach provided a simple and quantitative tool for assessing iron entry via facilitated diffusion or RME of Tf (22, 23), (ii) the efficacy and speed of action of iron chelators as scavengers of chelatable cell iron, and (iii) the status of chelatable cell iron, without apparently affecting the total cytosolic iron pools per se. Neither calcein nor specific iron(II) chelators such as BIP were found to significantly enhance conversion of iron(III) to iron(II) during measurements in aqueous solutions (Figs. 2, 4C, and 6). This would indicate that all fluorescence recovered by addition of BIP or other iron(II)-specific chelators to K562 cells represents actual intracellular iron(II). Results with other methods support that contention, however, they cannot unequivocally discern between a genuine intracellular iron(II) pool and a coexisting mixed pool of iron(II) and iron(III) which is very rapidly reduced upon addition of iron(II) chelators such as BIP. Such putative and extremely fast reduction of iron(III) might be compatible with the reducing conditions proposed to prevail in the cytosol of most cells (26). However, for reduction to occur intracellularly only when driven by iron(II) chelation is rather unlikely and is not supported by studies with calcein in solution. Also, iron acquired by RME of Tf showed qualitatively the chemical properties of iron(II), in agreement with previous studies (16, 21).

Iron(II) is most likely the form which is incorporated into the active form of the metal.
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