Effect of ATP depletion and temperature on the transferrin-mediated uptake and release of iron by BeWo choriocarcinoma cells

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We have recently described the transferrin-mediated uptake and release of iron by BeWo cells [van der Ende, du Maine, Simmons, Schwartz & Strous (1987) *J. Biol. Chem.* **262**, 8910–8916]. We now extend our studies of the mechanisms responsible for uptake and release of iron by these cells. Following preloading, ^{59}Fe release was maximal (about 12%) after about 4 h. Replacement of the extracellular medium with an equal volume of fresh medium either prior to or following the time at which equilibrium was reached further stimulated ^{59}Fe release. Both the rate and maximum amount of iron release decreased if longer loading times were used. Preincubation of BeWo cells for 15 min with 10 mM-sodium cyanide and 50 mM-2-deoxyglucose prior to the determination of ^{59}Fe release did not alter the amount released into medium (which did not contain a high-affinity iron chelator). However, under these conditions, the uptake of ^{59}Fe was dramatically inhibited as a result of prolongation of the transferrin–transferrin-receptor complex recycling time. These results demonstrate that the release of iron from BeWo cells is independent of cellular ATP levels, whereas iron uptake is ATP-dependent. Rates of both ^{59}Fe release and ^{59}Fe uptake were temperature-dependent. Analysis of these data via an Arrhenius plot suggests a single rate-limiting step for the release and uptake processes between 0 and 37 °C. The apparent energies of activation of these processes are very similar (approx. 59.0 kJ/mol for iron release and 50.6 kJ/mol for iron uptake), which raises the possibility that the release and uptake of iron share a common thermodynamically rate-limiting step. Possible mechanisms involved in iron release out of the cell and out of the endosome are discussed.

INTRODUCTION

All living cells, either prokaryotic or eukaryotic, require iron for growth. Iron is an essential component of many enzymes such as cytochromes, succinate dehydrogenase and iron–sulphur proteins, many of which function in the respiration chain. In multicellular organisms, iron is supplied to the cells by the serum protein ferrotransferrin. Transferrin is taken up by the cells via receptor-mediated endocytosis. The iron transport protein can bind either one or two iron atoms, and in its saturated form it has the highest affinity for its receptor at neutral pH 7.4. Receptor-bound ferrotransferrin is taken up by the cells via coated pits and coated vesicles (Aisen & Listowski, 1980; Young & Aisen, 1981), although there are suggestions that iron may also be taken up from transferrin prior to internalization (Thorstensen & Romslo, 1988). The internalized ferrotransferrin–receptor complex is transported intracellularly to a mildly acidic prelysosomal compartment in which the iron moiety dissociates from the polypeptide. Iron moves from this compartment into cellular stores, although the mechanisms are unknown. The apotransferrin–receptor complex remains intact at pH values below 6.5 and recycles to the plasma membrane

where it dissociates and the apotransferrin is released into the medium at neutral pH (van Renswoude *et al.*, 1982; Ciechanover *et al.*, 1983; Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). In addition to the predominant intracellular storage forms as ferritin, iron may accumulate into other minor storage pools (Jacobs, 1977; Bakkeren *et al.*, 1985; van der Ende *et al.*, 1987). However, the mechanism of iron transfer to these pools and the relationship between them is still poorly understood.

Cells which function in iron transfer must be able to release accumulated iron, as seen in the macrophage (Saito *et al.*, 1986), hepatocyte (Baker *et al.*, 1981; Mostert *et al.*, 1986) and Sertoli cell (Wauben-Penris *et al.*, 1988). The mechanism responsible for the release of iron by these cells is still unclear. Recently it was found that the release of iron by BeWo cells is not inhibited by the acidotropic drug primaquine or the ionophore monensin, and is stimulated by exogenous ligands (van der Ende *et al.*, 1987).

We have used the choriocarcinoma cell line BeWo of trophoblastic origin as a model system to study the mechanism(s) which underlie transferrin-mediated iron uptake and subsequent iron release. The BeWo cell line, as originally isolated by Pattillo & Gey (1968), exhibits

Abbreviations used: hCG, Human chorion gonadotropin; DTPA, diethylenetriaminepentaacetic acid; MEM, minimal essential medium; PBS, phosphate-buffered saline.

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many of the biochemical and morphological parameters of differentiation associated with the development of syncytiotrophoblast from cytotrophoblast. BeWo cells synthesize and secrete a spectrum of placental hormones including oestrogenic and progestational steroids, human placental lactogen and human chorionic gonadotropin (hCG). Exposure to methotrexate stimulates both alkaline phosphatase and hCG production; it also causes some degree of morphological differentiation, resulting in formation of multinucleated cells which appear to resemble the syncytiotrophoblast of the placenta (Speeg *et al.*, 1976; Friedman & Skehan, 1979). The syncytium is the physical barrier between the foetal and maternal blood circulations. Iron is transported unidirectionally across this cell layer, which possess an exceptionally high number of transferrin receptors on the microvilli. Iron is taken up in a transferrin-dependent manner (Faulk & Galbraith, 1979; Johnson *et al.*, 1980), but maternal transferrin does not cross the placenta (Gitlin *et al.*, 1964; Contractor & Eaton, 1986).

We have recently demonstrated the presence of a large number of transferrin receptors (3.5×10^6 per cell) and the rapid accumulation of iron in these cells. The accumulated iron is released as a low molecular mass complex via a process which can be stimulated by exogenously added iron chelators such as apotransferrin, desferrioxamine and diethylenetriaminepenta-acetic acid (DTPA) (van der Ende *et al.*, 1987).

In the present study we have further dissected the mechanism(s) of iron uptake and release by BeWo cells. Replacement of the extracellular medium with fresh medium caused an increase in ^{59}Fe release from preloaded cells. Moreover, the release is more efficient when shorter preloading times are used. The extent and kinetics of this process suggest that the release occurs from a small, rapidly releasable intracellular pool. The ^{59}Fe release process is not energy-dependent and occurs over a wide temperature range. However, while ^{59}Fe uptake is dependent upon cellular ATP, the effect of temperature on the rate of uptake is similar. The implications of these processes for transcellular iron movement are discussed.

MATERIALS AND METHODS

Cells

The b24 clone of the human trophoblast-derived cell line BeWo (Pattillo & Gey, 1968) (obtained from ATCC) was grown in Eagle's minimal essential medium (MEM) with 10% foetal calf serum as previously described (van der Ende *et al.*, 1987).

Materials

Iron-free human transferrin was purchased from Calbiochem, and Eagle's MEM and foetal calf serum were from Gibco. Na^{125}I and $^{59}\text{FeCl}_3$ were obtained from Amersham. 2-Deoxyglucose was from Sigma. Sodium cyanide was from BDH.

Labelling of transferrin

Transferrin was fully saturated with iron and radioiodinated with ^{125}I as described previously (van der Ende *et al.*, 1987) (specific radioactivity was generally $\sim 5 \times 10^8$ d.p.m./mg). Loading transferrin with ^{59}Fe was performed according to the procedure of van der Heul *et al.* (1978) with specific radioactivity $\sim 10^6$ d.p.m./mg.

Uptake of transferrin and iron

Uptake of ^{125}I -labelled ferrotransferrin and ^{59}Fe -ferrotransferrin was usually carried out in 60 mm dishes. Cells were incubated with 20 μg of transferrin/ml in 1 ml of binding medium (Eagle's MEM buffered with 20 mM-Na-Hepes, pH 7.4) at the temperature indicated. After incubation, the cells were washed with ice-cold phosphate-buffered saline (PBS; 170 mM-NaCl/3 mM-KCl/10 mM- Na_2HPO_4 /2 mM- KH_2PO_4) and dissolved in 1 M-NaOH as previously described (van der Ende *et al.*, 1987). The one-cycle experiments were carried out as described in detail previously (Ciechanover *et al.*, 1983; van der Ende *et al.*, 1987).

Cellular release of iron

The assay was essentially the same as described previously (van der Ende *et al.*, 1987). Cells were preloaded with ^{59}Fe by receptor-mediated endocytosis of ^{59}Fe -ferrotransferrin for 15 h. Thereafter, cells were rapidly washed three times with PBS at 0 °C followed by a 10 min incubation in sodium acetate buffer, (25 mM-sodium acetate/150 mM-NaCl/2 mM- CaCl_2) pH 4.5, at 0 °C and subsequently three incubations in binding medium for 30 min at 0 °C. After washing, the cells were incubated at 37 °C with prewarmed and pregassed (O_2/CO_2 ; 19:1) Eagle's MEM (with or without additions) for the times and under the conditions indicated in the text. At the appropriate times, samples of the media and/or cells were evaluated for ^{59}Fe .

During all experiments, cell viability was monitored by Trypan Blue exclusion.

RESULTS

Effect of extracellular media replacement on the release of iron

In an earlier study, we observed that the release of preloaded iron by BeWo cells was stimulated by the presence of exogenous iron chelators (van der Ende *et al.*, 1987). In the absence of added chelator, the release of iron into the extracellular medium (MEM) reached saturation after 2–4 h. Therefore, we examined the effect of replacement and composition of the medium on the release of iron. Cells were preloaded with ^{59}Fe -ferrotransferrin by overnight incubation. After extensive washing, the release medium (i.e. fresh MEM) was added to the cells and the kinetics of ^{59}Fe release were determined. As seen in Fig. 1, the release of ^{59}Fe is a process which can be described as pseudo-first order. At equilibrium (roughly 4 h), approx. 12% of the preloaded ^{59}Fe is released. Replacement of the extracellular medium with an equal volume of fresh medium either prior to or following the time at which equilibrium is reached (e.g. 180 or 300 min), further stimulated ^{59}Fe release as seen in Fig. 1. In addition, the total amount is proportional to the extracellular medium volume, with increased release seen with larger volumes (results not shown).

The presence of an iron chelator in the medium could easily explain these observations. Since various amino acids can serve as weak iron chelators (Schneider & Erni, 1982) we examined the effect of alterations in the composition of the medium on ^{59}Fe release. However, there was no increase in ^{59}Fe release in MEM supplemented with 2-fold essential amino acids when

Fig. 1. Effect of medium refreshment on the release of iron by BeWo b24 cells

Cells were preloaded with ^{59}Fe by transferrin-mediated endocytosis for 16 h. After an extensive wash procedure, cells were reincubated in MEM for the times indicated. At time points 180 min and 300 min, the medium was changed for fresh medium (A), while in the control plates the medium was not changed (B). After incubation, the cells were washed with PBS and subsequently dissolved in 1 M-NaOH.

compared to MEM alone (results not shown). Similarly, supplementation with 2-fold vitamins or 4-fold glucose failed to stimulate ^{59}Fe release (results not shown), whereas ^{59}Fe release is stimulated approx. 2-fold by the presence of exogenous chelator apotransferrin ($2.5\ \mu\text{M}$) (van der Ende *et al.*, 1987). Other potential iron chelators include both phosphate and citrate (Baker *et al.*, 1981; Mostert *et al.*, 1986; Andersson & Porath, 1986). Neither the absence or the presence of $1\ \text{mM-PO}_4$ or of $1\ \text{mM-citrate}$ (not normally present in MEM) altered ^{59}Fe release. All of these results taken together are consistent with the notion that in the absence of high-affinity iron chelator (e.g. $2.5\ \mu\text{M-apotransferrin}$), ^{59}Fe release from BeWo cells is diffusion-limited.

Additional characteristics of the pool available for rapid release were examined by evaluating ^{59}Fe release following different times of preloading. As seen in Fig. 2, the fraction of ^{59}Fe release decreases with the loading time, as well as the rate of release (Fig. 2b). These data support the presence of an intracellular ^{59}Fe pool which is rapidly loaded from external sources and from which iron is available for rapid release.

Effect of ATP depletion on iron release

The data presented above suggest that the iron release does not depend on cellular energy requirements. To investigate this, cells were incubated with sodium cyanide and 2-deoxyglucose in order to inhibit glycolysis and oxidative phosphorylation respectively and to deplete ATP levels. Following preloading of ^{59}Fe for 16 h, BeWo cells were incubated with $10\ \text{mM-sodium cyanide}$ and $50\ \text{mM-2-deoxyglucose}$ for 15 min prior to the de-

Fig. 2. Effect of the preloading time on the release of iron by BeWo cells

Cells were washed with PBS and preincubated for 45 min in MEM at 37°C . After preincubation, the cells were preloaded with ^{59}Fe by incubation with $20\ \mu\text{g}$ of [^{59}Fe]ferrotransferrin/ml for the times indicated. After the extensive wash procedure, the release was followed by incubation in MEM supplemented with 10% foetal calf serum (a). (b) $\log [(1 - B_t)/B_{\max}]$ plotted against the time, where B_t is the amount of ^{59}Fe released at time point t , and $B_{\max}$ is the maximal ^{59}Fe released (30 min preloading time). Correlation coefficients vary between 0.980 and 0.998. Preloading times: $\bullet$, 30 min; $\circ$, 60 min; $\blacksquare$, 120 min; $\square$, 300 min.

termination of ^{59}Fe release. As seen in Fig. 3(a), there was only a small (less than 10%) effect on ^{59}Fe release, when in MEM only. Additional experiments at concentrations up to $250\ \text{mM-sodium cyanide}$ in the presence of $50\ \text{mM-2-deoxyglucose}$ (results not shown)

Fig. 3. Effect of ATP depletion on the transferrin-mediated uptake and release of iron by BeWo cells

Cells were preincubated for 15 min with 50 mM-2-deoxyglucose and 10 mM-sodium cyanide prior to the determination of the release or uptake of iron by BeWo cells. (a) Release. After preincubation with the drugs, iron release by cells was measured in MEM containing the drugs. (b) Uptake. After preincubation with the drugs, [^{59}Fe]ferrotransferrin (20 $\mu\text{g}/\text{ml}$) was added to the medium and incubated for the indicated times. Non-specific ^{59}Fe uptake was determined by adding a 50-fold excess of unlabelled transferrin.

and 250 mM-2-deoxyglucose in the presence of 10 mM-sodium cyanide (Fig. 4a) confirmed the lack of effect (i.e. less than 12% inhibition) of these agents.

These results are in agreement with the results displayed in Fig. 1 and Fig. 2, and are consistent with the concept that in the absence of a high-affinity chelator, iron release from BeWo cells is diffusion-limited.

Fig. 4. Effect of 2-deoxyglucose concentration on the uptake and release of iron by BeWo cells and on the redistribution of the transferrin receptor

(a) Release (●) and uptake (○) of ^{59}Fe . Cells were preincubated for 15 min with 10 mM-sodium cyanide and increasing concentrations of 2-deoxyglucose prior to 4 h of ^{59}Fe uptake and release (for experimental details, see Materials and methods section). (b) Redistribution of transferrin receptor. Cells were preincubated for 60 min with 10 mM-sodium cyanide and increasing concentrations of 2-deoxyglucose in MEM + 10% foetal calf serum. Prior to 2 h of [^{125}I]ferrotransferrin binding at 0 °C (binding at cell surface), cells were freed from transferrin by an acid wash (pH 4.5) for 10 min and a neutral wash with MEM + 20 mM-Hepes, pH 7.4, for another 10 min. The total binding capacity was determined by binding of [^{125}I]ferrotransferrin to cells that were permeabilized by pretreatment with 0.1% saponin for 30 min.

Fig. 5. Effect of ATP depletion on one cycle of endocytosis of transferrin by BeWo b24 cells

Cells were preincubated in 50 mM-2-deoxyglucose and 10 mM-sodium cyanide in MEM for 15 min. After preincubation cells were incubated with 10 μ g of 125 I-ferrotransferrin/ml in binding medium at 0 °C for 2 h, whereafter they were washed three times with ice-cold PBS. The cells were then warmed to 37 °C by incubation in binding medium supplemented with 100 μ g of ferrotransferrin/ml. At the indicated times, medium was removed and the cells were washed with a sodium acetate buffer, pH 4.5, for 10 min and subsequently with binding medium for another 10 min, whereafter the cells were dissolved in 1 M-NaOH. The medium (●), the acid/neutral wash (indicating the surface bound transferrin, ○) and the cells in 1 M-NaOH (■) were counted separately in a gamma-counter.

Effect of ATP depletion on iron uptake

In order to determine which step in transferrin-mediated iron uptake is inhibited by ATP depletion and to confirm the depletion of ATP stores in BeWo cells under these conditions, we examined the effect of these agents on receptor-mediated endocytosis and uptake of 59 Fe from [59 Fe]ferrotransferrin during receptor recycling, as this process is energy-dependent (Ciechanover *et al.*, 1983; Clarke & Weigel, 1985; McAbbee & Weigel, 1987). As expected, the uptake of 59 Fe was dramatically inhibited in cells following incubation with 10 mM-sodium cyanide and 50 mM-2-deoxyglucose as seen in Fig. 3(b). Again, higher concentrations of up to 250 mM-sodium cyanide (+ 50 mM-2-deoxyglucose) (results not shown) and 250 mM-2-deoxyglucose (+ 10 mM-sodium cyanide) (Fig. 4a) did not further decrease the uptake of 59 Fe. The uptake in the presence of the inhibitors is about 25 % of the control level, most probably caused by a prolonged recycle time as observed with the asialoglycoprotein receptor (McAbbee & Weigel, 1987). Independent evidence in support of this is seen in Fig. 5, in which the effect of ATP depletion on a single cycle of 125 I-labelled ferrotransferrin uptake was examined. As demonstrated earlier, a single cohort of transferrin molecules loaded with iron enters cells, discharges iron to intracellular stores and recycles back to the extracellular medium (Ciechanover *et al.*, 1983; van der Ende *et al.*, 1987). However, as seen in Fig. 5, preincubation with sodium cyanide plus 2-deoxyglucose dramatically alters the normal ferrotransferrin cycle: 125 I-labelled ferrotransferrin leaves the cell surface with

the expected kinetics (half-time approx. 3–4 min, see Ciechanover *et al.*, 1983; van der Ende *et al.*, 1987). Extracellular transferrin accumulates from the cell-surface transferrin receptor with approx. 30 % remaining in the media, consistent with the well-described kinetics of transferrin–transferrin receptor dissociation at 37 °C (Ciechanover *et al.*, 1983). In addition, as expected, approx. 50 % of the cell surface transferrin moves into the BeWo cell (Fig. 5). However, this pool of transferrin molecules remains within the cell and is not able to (or at a far lower rate) recycle back to the extracellular medium. This observation is consistent with confirmatory data in Fig. 4(b). Here we see that a 2 h preincubation with increasing amounts of 2-deoxyglucose (0–100 mM) + 10 mM-sodium cyanide causes a depletion of the surface transferrin receptors (measured by 125 I-ferrotransferrin binding at 0 °C) to approx. 30 % of the control amount. The total number of transferrin receptors was measured by 125 I-ferrotransferrin binding at 0 °C but in the presence of 0.1 % saponin (Fallon & Schwartz, 1986). This detergent permeabilizes but does not solubilize the cell membrane, enabling macromolecules as large as 2×10^5 kDa to enter the cell (Shepherd *et al.*, 1984). Saponin does not cause the loss of membrane-bound molecules (Simmons & Schwartz, 1984). As seen in Fig. 4(b), the total number of transferrin-binding sites remains virtually the same with increasing concentrations of 2-deoxyglucose. This means that in the presence of 2-deoxyglucose and sodium cyanide, only 10 % of the transferrin receptors are at the cell surface instead of the normal 40 %. Fig. 5 shows that the endocytosis rate is normal compared to control cells. To maintain the distribution of the transferrin receptors in drug-treated cells, the externalization rate has to be significantly reduced, and hence the recycle time of the receptor increased (Ciechanover *et al.*, 1983). This is in fact observed: Fig. 5 shows that during the course of the experiment, no visible reduction of the intracellular transferrin is observed, while Fig. 3(b) shows a more than 4-fold reduction in iron uptake. In conclusion, iron uptake via the transferrin–transferrin-receptor cycle in BeWo cells is dependent upon cellular ATP levels. In this cycle the rate of endocytosis is normal, whereas the externalization is inhibited.

Effect of temperature on iron uptake and release

In order to further characterize the processes involved in the release of 59 Fe from BeWo cells, we examined the effect of temperature on both 59 Fe release and 59 Fe uptake. As seen in Fig. 6, the rate of 59 Fe release is temperature-dependent, with increases in the rate of release with elevated temperature. Analysis of the data using the Arrhenius equation (see Fig. 6b) demonstrates a linear relationship between the logarithm of the rate of 59 Fe release and 1/temperature in degrees Kelvin. This suggests a single rate-limiting step for the release process between 0 and 37 °C. From these data, the apparent energy of activation of this process is approx. 59.0 kJ/mol. Similarly, from an analysis of the effect of temperature on the rate of 59 Fe uptake in these same cells, the apparent energy of activation of the uptake process is approx. 50.6 kJ/mol (Fig. 6). The value of 59.0 kJ/mol for the apparent energy of activation for the uptake of 59 Fe via transferrin-mediated endocytosis is in close agreement with the value found for the endocytosis of asialo-orosomucoid (Weigel & Oka, 1985) but sub-

Fig. 6. Effect of temperature on the uptake and release of transferrin and iron by BeWo b24 cells

For uptake measurements (○), cells were preincubated in MEM at 37 °C for 60 min and were then incubated for 4 h at the indicated temperature with [^{59}Fe]ferrotransferrin in binding medium. After incubation, the cells were washed with binding medium three times and subsequently dissolved in 1 M-NaOH. Release (●) of iron at different temperatures was determined as follows. Cells were preloaded with ^{59}Fe as indicated in the legend of Fig. 1. After washing, the cells were reincubated in binding medium at the indicated temperatures for 4 h. (a) Rate of uptake of release versus temperature. (b) Arrhenius plot, in which log rate is plotted against $1/\text{temperature}$ in K. The correlation coefficients are $r = 0.979$ (uptake) and $r = 0.995$ (release).

stantially lower than the values reported by others for the endocytosis of several other proteins (Steinman *et al.*, 1974; Kaplan & Nielsen, 1979; Dunn *et al.*, 1980).

DISCUSSION

In an earlier study, we described that BeWo cells can release previously accumulated iron in significant quantities, whereas the cervical epithelial HeLa cells do not show significant release of their accumulated iron (van der Ende *et al.*, 1987). The release of iron by BeWo cells is stimulated by exogenously added chelators such as apotransferrin, lactoferrin, DTPA and desferrioxamine. Lactoferrin, DTPA (unable to enter the cell) and desferrioxamine (able to enter the cell, Bottomley *et al.*, 1985) are more or less competitive with apotransferrin in chelating the released iron. Moreover, when no chelators are present in the medium, the released iron has a low molecular mass form. The nature of this complex is unknown. It elutes in the included volume of a Sephadex G-100 gel-filtration column and is able to pass through a filter with a 30 kDa molecular mass cutoff (van der Ende *et al.*, 1987). Here we show that the release of iron by BeWo cells is dependent on replacement of the release medium (a limiting component in the medium was excluded by varying the composition of the release medium). Also, the extent of iron release appeared to depend on the time of iron uptake, i.e. the shorter the time of iron uptake the more efficiently intracellular iron is released. These results, together with the earlier data, are interpreted as follows. Iron is taken up by the cell and intracellularly dissociated from transferrin in an acidic compartment (van Renswoude *et al.*, 1982; Dautry-Varsat *et al.*, 1983). The iron atoms are then transferred in an unknown way to a cytosolic pool which contains the iron in a low molecular mass form. From this pool, iron can be transferred to cytosolic ferritin for long-term storage or trafficked to the external medium. From the

low molecular mass pool, the iron is rapidly released to the external medium until an equilibrium is reached across the plasma membrane. A rapidly releasable pool of iron has also been observed using rat Sertoli cells (Wauben-Penris *et al.*, 1988), rat hepatocytes (Octave *et al.*, 1983) and macrophages (Saito *et al.*, 1986). Release from the ferritin (high molecular mass) pool is much slower and occurs via the intracellular pool of rapidly releasable iron. This pool is in equilibrium with cytoplasmic ferritin (van der Ende *et al.*, 1987), but can be stimulated by adding external chelators, which remove the low molecular weight iron from the medium. Release of iron, uncoupled from endocytosed transferrin, has also been observed recently in experiments *in vivo* using the perfused human placenta lobule. It was shown that maternal transferrin is not transferred, but that iron is transferred to the foetal circulation where it becomes associated with foetal transferrin (Contractor & Eaton, 1986).

The uptake of iron by BeWo cells was severely inhibited following ATP depletion. Single-cycle experiments showed that the internalization was not inhibited. However, the externalization rate for apotransferrin appeared to be dramatically decreased and might even be completely inhibited. The decrease in the number of transferrin-binding sites from the cell surface after cellular ATP depletion confirmed this notion. This appears to be a general property of receptor-mediated endocytosis, as similar results have been observed for the asialoglycoprotein receptor (Clarke & Weigel, 1985; McAbbee & Weigel, 1987). These studies demonstrated that a single cycle of endocytosis of the asialoglycoprotein receptor was not influenced by ATP depletion, whereas the continuous recycling of this receptor was inhibited. In addition, the asialoglycoprotein receptor does not return to the cell surface, with loss of cellular ATP. Evidently the first steps in endocytosis (pinching off coated vesicles) need far less energy than later steps

such as uncoating of the vesicles and acidification of endosomes. The results of studies *in vitro* are in agreement with these observations: energy required for pinching off the coated vesicles is driven by the free-energy change associated with the self assembly of clathrin, whereas ATP is needed to uncoat coated vesicles (Schmid *et al.*, 1984; Moore *et al.*, 1987). Moreover, acidification of endosomes requires ATP-driven proton pumps (Forgac *et al.*, 1983; Stone *et al.*, 1983; Yamashiro *et al.*, 1983).

The last step in iron release from BeWo cells is the transfer of a low molecular mass iron component across the plasma membrane. This process may be comparable to the release of iron atoms out of the acidic endosome during transferrin-mediated iron uptake, as the iron uptake and release show nearly identical dependence on temperature. The energies of activation obtained from the respective Arrhenius plots are in close approximation. Given the many assumptions which must be taken into account in any determination of apparent energies of activation of rate-limiting steps in overall processes (e.g. that the substrate for the overall process is saturating at all temperatures, etc.), it is surprising that the effects of temperature on these two processes are so similar. This raises the issue as to whether the two processes share a common thermodynamically rate-limiting step. Specifically, the steps in which iron has to pass the membrane are most likely similar, i.e. when it moves out of the endosome into the cytosol and out of the cytosol into the medium respectively. How the iron atoms are transferred out of the acidic compartment after dissociation from transferrin during iron uptake and out of the cytosol during iron release is still unclear. Iron is bound to transferrin in the Fe(III) form. Release of iron [in the Fe(III) form] from transferrin can be established by lowering the pH to about 5.5, as occurs in the mildly acidic endosome within the cell (van Renswoude *et al.*, 1982; Dautry-Varsat *et al.*, 1983). Thereafter, the iron atoms have to be transferred to the cytosol. This may occur via the Fe(II) form. This is also the form of iron which enters cytosolic ferritin, after which the iron is oxidized to the Fe(III) form [for a recent overview see Crichton & Charlotte-Wauters (1987)]. Indeed, a transferrin reductase which is apparently associated with the transferrin receptor has been implicated in transferrin-mediated iron uptake, and might convert ferric iron released in the acidic compartment to ferrous iron (Sun *et al.*, 1987). As seen above, iron which has entered the cytosol may have two fates (i.e. to ferritin for long term storage, or to the medium upon release). The form in which iron is released is unknown, but is chelatable by apotransferrin, DTPA and desferrioxamine. Apotransferrin binds only ferric iron [but can accept both ferric iron and ferrous iron; the latter is oxidized by the protein; Koijoma & Bates (1981)]. However, DTPA and desferrioxamine are specific Fe³⁺ chelators. Thus, either the iron in the cytosol is in the Fe(III) form, or is converted to the Fe(III) form in the extracellular medium, e.g. when transferred across the plasma membrane.

Remarkably, the release of iron out of the cytosol into the external milieu is not inhibited by ATP depletion. The results obtained with replacement of the release medium support this. Moreover, we have previously shown that the release of iron by BeWo cells is not inhibited by primaquine or monensin, while iron uptake was inhibited under the same conditions, suggesting that

vesicular transport is not involved in the release of iron (van der Ende *et al.*, 1987). One attractive possibility is that the redox reaction of Fe(II)/Fe(III), which is linked to membrane-located electron transport, is the driving force in the release of iron from cells. Another possibility is that a carrier-mediated mechanism is operative in iron transport into the cytoplasm, as well as in iron release out of the cytoplasm and into the extracellular medium. Further studies will be necessary to compare both processes and resolve their mechanisms.

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