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Involvement of Transferrin in the Reduction of Iron by the Transplasma Membrane Electron Transport System

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Abstract

Nonpermeable electron acceptors can be reduced by a transplasma membrane electron transport system in suspensions of intact cells. Here we report that diferric transferrin is reduced by HeLa S3 cells. The reduction is recorded spectrophotometrically as the formation of the ferrous complex of bathophenanthroline disulfonate. Ferric ammonium citrate can also be used as an electron acceptor, and the presence of low concentrations of diferric transferrin greatly stimulates the reduction of trivalent iron under these conditions. Likewise very low concentrations of ferricyanide, which does not give rise to a ferrous bathophenanthroline disulfonate complex formation, have a strong stimulatory effect on the complex formation when ferric ammonium citrate is the source of ferric iron. Apotransferrin is a potent inhibitor of the reaction. The inhibition occurs at the concentration necessary for complete occupancy of the transferrin receptors. The inhibition can be demonstrated also when high concentrations of ferricyanide are used as electron acceptor. The possible mechanism behind the reported phenomena is discussed, and it is concluded that the transplasma membrane electron transport system can be involved in the process of cellular iron uptake.

Key Words: Plasma membrane oxidoreductase; transferrin; transferrin receptor; iron transport; cell growth.

Introduction

Diferric transferrin is the principal iron transport protein in the blood. Two opposing views have developed concerning the mechanism of iron transfer

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from the transferrin across the plasma membrane into cells. One proposal involves release of iron from transferrin bound at the plasma membrane and transport of the iron through the plasma membrane (Nunez *et al.*, 1983; Thorstensen and Romslo, 1984; Cole and Glass, 1983; Morley *et al.*, 1982, 1984). The other envisions receptor-mediated endocytosis of the iron transferrin attached to transferrin receptors into endosomes where the iron is released in the acid environment for transport through the endosome membrane (Morgan and Appelton, 1969; May and Cuatrecasas, 1985; Hanover and Dickson, 1985) into the cytoplasm. Release of iron from transferrin for entry into the cell may be achieved either by acidification (Baldwin *et al.*, 1982; Harding and Stahl, 1983; Carver and Frieden, 1978) or by reduction to ferrous iron (Carver and Frieden, 1978; Harris *et al.*, 1985; Gaber and Aisen, 1970; Aisen, 1980; Kojima and Bates, 1979; Hagen, 1982). Based on studies of effects of ferrous iron chelators in iron uptake, reduction either at the cell surface (Nunez *et al.*, 1983; Thorstensen and Romslo, 1984; Cole and Glass, 1983) or in endosomes (Morgan, 1983a) has been proposed. At the cell surface the pH is slightly alkaline so iron release by acidification is unlikely unless local protonation associated with the transferrin receptor occurs (Carver and Frieden, 1978). In endosomes the acid pH would favor dissociation of ferric iron from the transferrin (Octave *et al.*, 1983). The free ferric iron could easily be subsequently reduced by common reducing agents or by enzymes.

The direct chemical reduction of iron in diferric transferrin is very difficult to achieve with common biological reducing agents such as ascorbate, glutathione, or NADH (Carver and Frieden, 1978; Harris *et al.*, 1977; Egyed, 1973; Sommer *et al.*, 1982). Significant reduction has been achieved only in the presence of millimolar concentrations of pyrophosphates (Carver and Frieden, 1978; Konopka *et al.*, 1981).

Hemoglobin, or methemoglobin plus NADH, in the presence of pyrophosphates has been shown to mediate a slow reductive release of iron from transferrin (Egyed *et al.*, 1980; Egyed, 1975), but the rate of release is much faster with intact reticulocytes (Egyed, 1973; Aisen and Leibman, 1973; Williams and Woodworth, 1973; Martinez-Meddellin and Schulman, 1973). Several studies have shown that ferrous iron can be formed and released from diferric transferrin in the presence of intact cells such as reticulocytes or hepatocytes (Thorstensen and Romslo, 1984; Egyed *et al.*, 1980; Jandl *et al.*, 1959). The location of the iron release is difficult to establish when lipophilic chelators for ferrous iron are used to detect the reduced iron (Morgan, 1983a). Chelators such as dipyridyl and *o*-phenanthroline form iron complexes which remain associated with membranes. Thorstensen and Romslo (1984), by using bathophenanthroline disulfonate,[4] which is water soluble

[4]Abbreviations used: BPS, bathophenanthroline disulfonate; BSA, bovine serum albumin.

and impermeable to membranes, showed that more than 50% of the ferrous bathophenanthroline disulfonate complex formed during incubation of hepatocytes with iron transferrin remains in the supernatant after removal of the cells. These authors also showed that the chelator does not extract iron from cells after it has been taken up (Thorstensen and Romslo, 1984). Thus there is evidence for reductive removal of iron from transferrin at the cell surface.

The iron bound to transferrin is released at acidic pH in the ferric form. After release from transferrin, the ferric iron is more easily reduced to the ferrous state by reducing agents or enzymes (Carver and Frieden, 1978). Iron uptake in cells has been shown to occur by internalization of iron transferrin bound to transferrin receptors into endosomes (Morgan and Appelton, 1969; May and Cuatrecasas, 1985). The ferric iron can then be released in the endosomes, where the pH is 5.4, and the apotransferrin can be returned to the cell surface and released from the receptor (Cole and Glass, 1983). Since permeable ferrous iron chelators interfere with iron uptake by cells, the ferric iron released in the endosome is apparently reduced to ferrous iron before entering the cytoplasm through the endosome membrane (Morgan, 1983a).

In this paper we describe a transplasma membrane electron transport system which, in conjuction with the transferrin receptor, reduces diferric transferrin at the cell surface. It has previously been shown that many cells, even those with few transferrin receptors such as erythrocytes (Lodish and Small, 1975), can reduce impermeable oxidants such as ferricyanide (Grebing *et al.*, 1984) and that this reduction is associated with proton release at the cell surface (Sun *et al.*, 1984b). Diferric transferrin can be used as electron acceptor for this electron transport system with the release of external ferrous iron. The reduction of the diferric transferrin depends on the presence of the trasferrin receptor. A preliminary report of part of these results has been presented at the American Society for Cell Biology meeting in 1985 (Crane *et al.*, 1985).

Methods

Growth of HeLa S3 Cells

HeLa S3 cells were grown in suspension in Eagle's minimum essential medium for suspension cultures supplemented with 10% fetal bovine serum, benzyl penicillin (50 μg/ml), and streptomycin (50 units/ml) in a humidified atmosphere of 95% O_2 and 5% CO_2. Medium was changed every second day. Cell viability was monitored by trypan blue exclusion.

Transferrin Preparations

Diferric transferrin was prepared from human apotransferrin (Sigma, St. Louis, Missouri) according to Karin and Mintz (1981). Only preparations with an absorbancy ratio 465 nm/280 nm of 0.043 or higher were used. Alternatively a commercial preparation of human diferric transferrin (Miles laboratories, Naperville, Illinois) was used. The preparation had an absorbancy ratio 465 nm/280 nm of 0.043.

Apotransferrin was iodinated according to the lactoperoxidase method, and the iodinated peptide was separated on a Sephacryl S 200 column (Thorell and Johansson, 1971; Gliemann *et al.*, 1979).

Binding Procedure

The binding studies were performed in polystyrene tubes coated with BSA. Each tube contained 1.9×10^6 cells, iodinated apotransferrin (approximately 0.3 μCi ^{125}I in 0.16 ng apotransferrin), and increasing concentrations of unlabelled apo- or diferric transferrin, in a final volume of 300 μl. The incubation was carried out at 37°C for 15 min and the incubation was stopped by separating the cells from the incubation media by centrifugation of 100-μl aliquotes at 12,000 $\times$ *g* for 30 sec, passing the cells through a mixture of phthalic acid dibutyl ester and phthalic acid dinonyl ester (3 : 2). The incubation medium and part of the hydrophobic layer were aspirated and the tip of the tube containing the cells cut off and counted for its content of ^{125}I.

Reduction of Iron

The reduction of ferric ions was measured by following the formation of ferrous bathophenanthroline disulfonate complex. Light absorbance was measured at 535 nm against 600 nm with a dual-beam spectrophotometer (Shimadzu UV 300). The extinction coefficient used for ferrous bathophenanthroline disulfonate at 534–600 nm was 17 $cm^{-1}mM^{-1}$ (actual value 17.6 $cm^{-1}mM^{-1}$). The incubation medium was a salt solution with 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ in 10 mM Hepes buffer, pH 7.4. this medium was also used in the binding experiments. In the spectrophotometric experiments the final volume was 2.5 ml and the reaction was started by the addition of the various ferric complexes. The cell density was 0.75–1.5 $\times$ 10^6 cells per 2.5 ml and the incubation temperature was 37°C.

Results

Reduction of Diferric Transferrin

Incubation of HeLa S3 cells with low concentrations of diferric transferrin in the presence of the iron chelating agent bathophenanthroline disulfonate

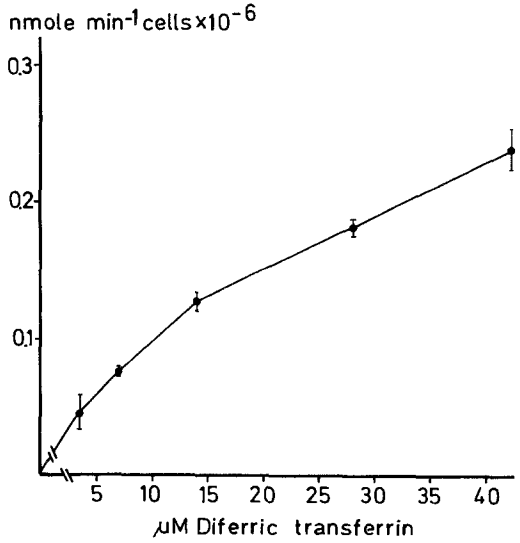

Fig. 1. Rates of ferrous BPS complex formation with various concentrations of diferric transferrin in the presence of 15 μM BPS. mean of six experiments with SEM indicated. Experimental conditions in Methods section.

Table I. Formation of BPS Ferrous Complex in the Presence of HeLa S3 Cells with Diferric Transferrin, Ferric Ammonium Citrate, and BPS[a]

Components added	Initial rate of BPS ferrous complex formation (nmol min^{-1} cells $\times$ 10^{-6})
Cells, 28 μM diferric transferrin, 7.5 μM ferric ammonium citrate, 15 μM BPS	2.74
– ferric ammonium citrate	0.54
– diferric transferrin	1.62
– cells	0

[a]Conditions in Methods section.

leads to the formation of BPS ferrous complex with an absorption maximum at 535 nm. Figure 1 shows the rate of increase of the absorbance at 535 nm, using 600 nm as the reference wavelength, observed with various concentrations of diferric transferrin under these conditions over 6 min. The iron reduction observed is dependent on the presence of cells, diferric transferrin, and BPS (Table I). Values given are initial rates. Apotransferrin cannot replace diferric transferrin in the assay.

HeLa S3 cells will also reduce other ferric chelates such as ferric ammonium citrate (Table I). Addition of 7 μM diferric transferrin increases the reduction rate in the presence of ferric ammonium citrate up to a concentration of 60 μM. The presence of trace amounts of ferric ammonium

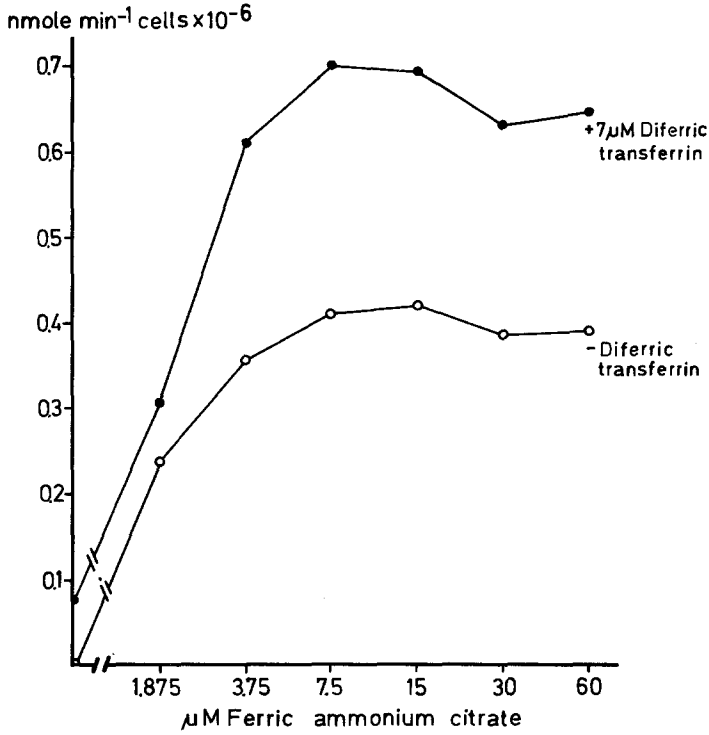

Fig. 2. Effect of ferric ammonium citrate concentrations on the rate of ferrous BPS complex formation in the presence of 15 μM BPS, with and without 7 μM diferric transferrin. Experimental conditions in Methods Section.

citrate in the diferric transferrin therefore would not account for the enhancement of activity with diferric transferrin (Fig. 2). Saponin (2%) and Triton X-100 (0.2%) inhibit ferric ammonium citrate reduction, which shows that intact cells are required (not shown).

The rate of iron reduction increases with increasing HeLa S3 cell concentration up to 1.5 million cells per 2.5 ml of the Hepes-salts solution (Table II).

The rate of BPS ferrous complex formation increases with both increasing ferric ammonium citrate concentrations and increasing diferric transferrin concentrations. The concentration of ferric ammonium citrate for half maximal stimulation of iron reduction is found at 2.5 μM. The same concentration gives half maximal stimulation in the presence of 7 μM diferric transferrin (Fig. 2). The concentration of diferric transferrin for half maximal stimulation of the reduction rate is approximately 20 μM. Since serum contains up to 40 μM transferrin (Bezkorovainy and Zschocke, 1975), a sufficient concentration is present to maintain a high rate of reduction.

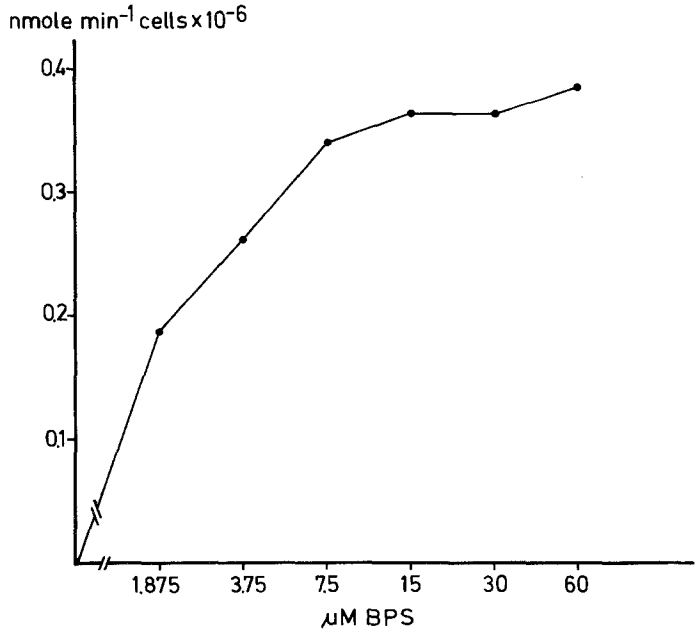

Fig. 3. Effect of BPS concentrations on the rate of ferrous BPS complex formation with 7.5 μM ferric ammonium citrate as a substrate. Experimental conditions in Methods section.

Table II. Diferric Transferrin Stimulation of the Rate of BPS Ferrous Complex Formation at Different Cell Concentrations[a]

	Rate of BPS ferrous complex formation (nmol min^{-1})	
	No diferric transferrin added	14 μM diferric transferrin added
No cells	0.0	0.0
0.75 × 10^6 cells	0.29	0.45
1.5 × 10^6 cells	0.61	0.76

[a]Conditions in Methods section. Ferric ammonium citrate at 7.5 μM in all assays.

The concentration of BPS necessary to approach maximum activity is 15 μM with 7.5 μM ferric ammonium citrate as the electron acceptor (Fig. 3). There is no inhibition of ferric ammonium citrate reduction by BPS in concentrations up to 60 μM.

Apotransferrin Inhibition

The rate of BPS ferrous complex formation with diferric transferrin in the presence and absence of apotransferrin is shown in Fig. 4. The effect of apotransferrin on ferric ammonium citrate reduction is shown in Fig. 5.

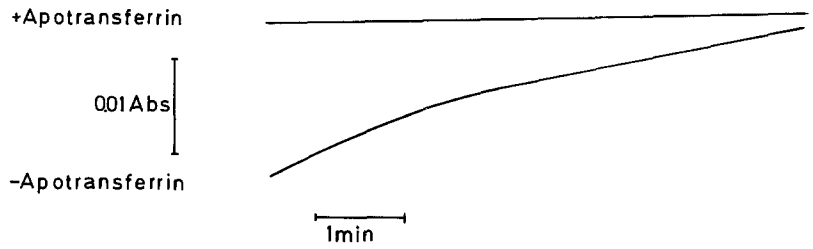

Fig. 4. Tracing of absorbance difference 535–600 nm of BPS ferrous complex formed with 28 μM diferric transferrin in the presence and absence of 3.5 μM apotransferrin. The cell density was 0.3 $\times$ 10^6 ml^{-1}. Experimental conditions in Methods section.

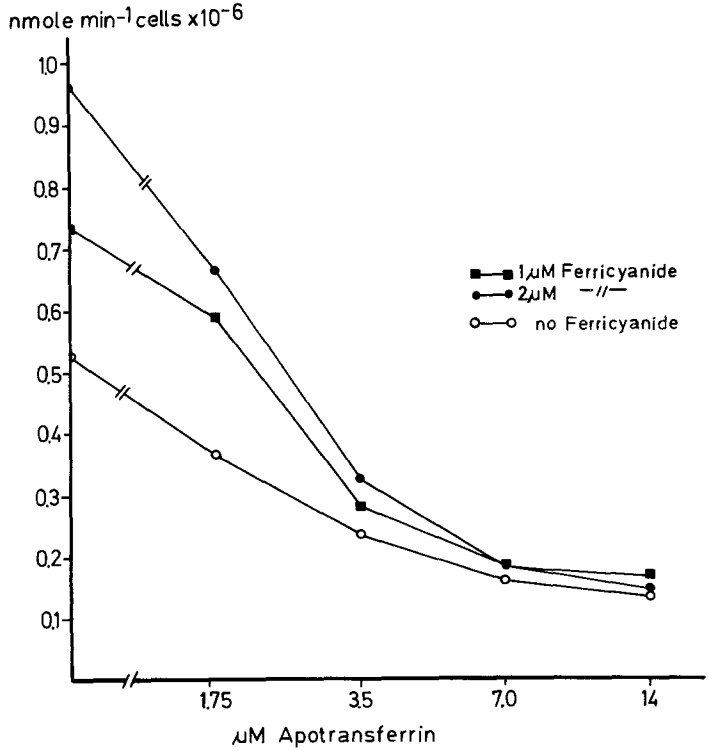

Fig. 5. Apotransferrin inhibition of ferric ammonium citrate reduction with and without ferricyanide. Open circles: no ferricyanide present. Filled squares: 1 μM potassium ferricyanide. Filled circles: 2 μM potassium ferricyanide. Experimental conditions in Methods section.

Apotransferrin inhibits the reduction of both diferric transferrin and ferric ammonium citrate. Maximal inhibition of ferric ammonium citrate is observed at 7 μM apotransferrin.

Reduction of Ferricyanide

Reduction of external ferricyanide by HeLa S3 cells has been demonstrated previously (Sun *et al.*, 1984b). A 30% inhibition of this transplasma

Table III. Inhibition of Ferricyanide Reduction by HeLa S3 Cells with Apotransferrin[a]

	Initial rate of ferricyanide reduction (nmole min^{-1} cells $\times$ 10^{-6})
0.2 mM Ferricyanide	3.98
0.2 mM Ferricyanide + 7 μM apotransferrin	2.75

[a]Ferricyanide reduction was measured as decrease of the absorbance difference between 420–500 nm in a dual-beam spectrophotometer. Conditions in Methods section.

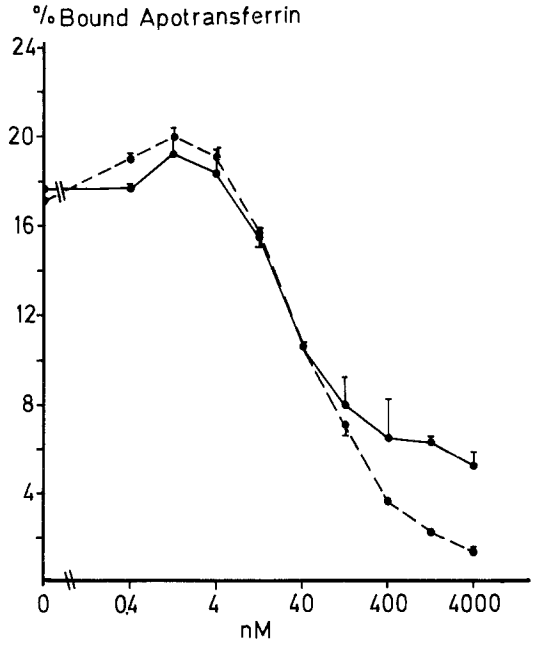

Fig. 6. Displacement of ^{125}I-apotransferrin by diferric and apotransferrin from intact HeLa S3 cells. Solid line: apotransferrin; broken line: diferric transferrin. Experimental conditions in Methods section.

Table IV. Effect of pH on the Binding of Apotransferrin to HeLa S3 Cells.[a]

Conditions	Percent binding
Hepes, pH 7.4	19.5 ± 0.3
Pipes, pH 7.4	18.3 ± 0.1
pH 6.2	17.4 ± 1.3

[a]Experimental conditions in Methods section.

membrane electron transport is noted in the presence of 7 μM apotransferrin, which indicates that the electron transport to ferricyanide involves the same system as the transferrin reduction (Table III).

The relation between the ferricyanide reduction and that of transferrin iron is further indicated by the strong stimulation of ferric ammonium citrate reduction by micromolar concentrations of ferricyanide (Fig. 5). Apotransferrin inhibits the ferricyanide-induced reduction of ferric ammonium citrate at concentrations that are the same as for inhibition of transferrin iron reduction (Figs. 4 and 5). This shows that the ferricyanide reduction is closely related to the transferrin reduction system and that low levels of ferricyanide allow a more complete inhibition than the high concentrations of ferricyanide used in Table III.

Transferrin Binding

Apotransferrin binds to HeLa S3 cells, and ^{125}I-labelled apotransferrin is displaced both by diferric transferrin and apotransferrin, with half maximal displacement at 25 nm for diferric transferrin as well as for apotransferrin (Fig. 6).

With HeLa S3 cells, apotransferrin binding is observed at both acidic and neutral pH with only a slight decrease in apparent affinity at pH 6.2 (Table IV).

Discussion

Transplasma membrane electron transport to external ferricyanide has been demonstrated for many cells including HeLa S3 cells (Grebing *et al.*, 1984; Sun *et al.*, 1984b). The present findings suggest that iron transferrin is the natural electron acceptor for this enzyme system. There is an initial rapid rate of formation of BPS ferrous complex in the presence of HeLa S3 cells and of diferric transferrin, which then declines. If ferric ammonium citrate is added together with diferric transferrin, the rate is increased over the rate observed with ferric ammonium citrate alone as electron acceptor.

The declining rate of reduction observed with diferric transferrin (Fig. 4) as the reducible substrate can be explained by a sustained binding of the apotransferrin after the iron of diferric transferrin has been reduced and released as ferrous ions complexed by BPS. This product inhibition indicates that the apotransferrin inhibition of diferric transferrin binding is even greater than is indicated by the binding studies. These studies show that bound apotransferrin can be displaced by both apotransferrin and diferric transferrin in equal concentrations.

Apotransferrin inhibits iron reduction in diferric transferrin present at 10-fold the concentration of apotransferrin. The reduction is assayed in the presence of a chelator of the ferrous iron, and thereby a reformation of an iron transferrin complex becomes unlikely. Low concentrations of apotransferrin have also been reported to inhibit uptake of iron from diferrin transferrin by hepatocytes (Young and Aisen, 1980).

Inhibition of diferric transferrin reduction occurs at the same concentration of apotransferrin as required for complete displacement of bound radioactive apotransferrin. When ferric ammonium citrate, or ferricyanide together with ferric ammonium citrate, are the electron acceptors the formation of BPS ferrous complex is not completely inhibited, whereas the reduction of diferric transferrin alone is completely inhibited by apotransferrin. This suggests that a part of the reduction of the iron chelates is not mediated via a transferrin receptor-related oxidoreduction.

Ferricyanide is found to catalyze ferric iron reduction at remarkably low concentrations. Micromolar concentrations of ferricyanide give a 100% stimulation of the formation of BPS ferrous complex. On the other hand, if corresponding concentrations of ferricyanide are present alone, little or no reduction can be observed. Concentrations in the millimolar range are required when ferricyanide is used as the only electron acceptor (Sun *et al.*, 1984b).

The catalytic effect of ferricyanide on ferric ammonium citrate reduction may be explained by the high negative charge of the ferricyanide being a prerequisite for its reaction with the catalytic site. Reduction results in a more negative anion, and a tighter binding of the ferrocyanide iron. This in turn results in a product inhibition of the reaction as previously observed for liver cells (Clark *et al.*, 1981). The reaction is thus accelerated when the bound ferrocyanide is reoxidized by the added ferric ammonium citrate.

The catalytic effect of diferric transferrin on the ferric ammonium citrate reduction can be explained in a similar way. The diferric transferrin is bound to the receptor. The apotransferrin is retained on the receptor after reduction and the concomitant release of the iron. The presence of ferric ions in the form of the ferric ammonium citrate complex provides ferric iron for reformation of a diferric transferrin complex on the transferrin receptor.

The redox potential of diferric transferrin in buffer has been reported to be either −400 mV (Harris *et al.*, 1985) or −140 mV (Harris, 1983). HeLa S3 cells have been shown to reduce impermeable oxidants which have a midpoint redox potential as low as −150 mV (Sun *et al.*, 1984a). Therefore the plasma membrane redox system could reduce diferric transferrin, if its redox potential when attached to the transferrin receptor is −150 mV or higher. Sommer *et al.* (1982) propose that the reduction of diferric transferrin is controlled by a low rate constant determined by diferric steric properties and that distortion of the diferric transferrin is necessary for reduction.

Since glutathione and cysteine do not reduce ferric iron bound to transferrin (Egyed, 1973), it is apparent that the iron site is well protected from external reductants. Attachment to the transferin receptor may change the redox potential or the iron binding control for the transferrin.

The binding of apotransferrin to the receptor at pH above 7.0 in HeLa S3 cells differs from the reported lack of binding at neutral pH observed with reticulocytes (Morgan, 1983b; Nunez and Glass, 1983). The release of apotransferrin at pH 7.0 and above from reticulocytes is important for the operation of the iron transport cycle. The transport cycle depends on binding of apotransferrin to the receptor inside the acidic endosome, to return the apotransferrin to the surface, where it is released at neutral pH (Hanover and Dickson, 1985; Morgan, 1981, 1983b). It appears that with HeLa S3 cells the reduction of iron at the surface is favored over the iron uptake cycle, although a recycling of apotransferrin also has been observed with HeLa S3 cells (Morgan, 1981). Transferrin has been shown to concentrate on the surface of HeLa S3 cells where exocytosis is most active (Bretscher, 1983). Transferrin receptor concentration on the surface of HeLa S3 cells has been shown to be controlled by conditions which modify heme content. Hemin decreased transferrin receptors more than ferric ammonium citrate (Ward *et al.*, 1984). The effects of changes in surface exposure of transferrin receptors on the rate of diferric transferrin reduction remains to be studied.

We emphasize that the meaning of the transplasma membrane transferrin reductase in relation to iron uptake should not be interpreted hastily. The electron transport could be used to facilitate iron uptake either at the surface or from endosomes, on the basis that endosome membrane derives from plasma membrane and thus will contain the redox system. The endosome membrane electron transport system could also use cytosolic NADH to reduce the ferric iron liberated from the transferrin by the acidic pH inside.

The results presented show that diferric transferrin can act as a natural electron acceptor for the transplasma membrane electron transport system in mammalian cells. Transferrin is known to stimulate the growth of many cells in the absence of fetal calf serum (Barnes and Sato, 1980). It has also been

found that other artificial electron acceptors, even of nonferric type (Sun *et al.*, 1984a), can stimulate growth via the transplasma membrane oxidoreduction system. Part of the growth promotion by transferrin may therefore derive from its action as an external electron acceptor. This would be consistent with the observation that the most effective form of transferrin for growth stimulation is the diferric form (Huebers *et al.*, 1983). Inhibition of interleukin-dependent DNA synthesis by the 42/6 antibody for transferrin receptors may be based on inhibition of diferric transferrin reduction similar to the effect of apotransferrin observed with HeLa cells (Neckers and Cossman, 1983). The contrasting effects of several monoclonal antibodies on growth stimulation (Taetle *et al.*, 1983) and iron uptake (McArdle and Morgan, 1984) induced by transferrin strongly suggests two separate mechanisms. The monoclonal antibodies 42/6, B3/25, and 43/31 inhibit myeloid cell growth, whereas they stimulate iron uptake.

In their discussion of the mechanism of transferrin uptake by cells, Octave *et al.* (1983) state: "A convincing mechanism which could permit transferrin to release its iron at the plasma membrane is conspicuous by its absence". We suggest that the reduction of diferric transferrin by the transplasma membrane electron transport in conjunction with the transferrin receptor provides a viable approach to reductive release at the plasma membrane and even provides a source of protons to assist in this release. Whether the reduction and release at the plasma membrane favors or prevents iron uptake into a given cell type remains to be established.

Since diferric transferrin can act as electron acceptor for the redox system, and is important for growth stimulation by many cells, the external reduction of the diferric transferrin may be important for cell growth rather than for iron uptake only. As pointed out by May and Cuatrecasas (1985): "One mechanism by which transferrin . . . could possibly initiate growth-related transmembrane signaling, which could be unrelated to iron delivery, would be by activating a transferrin receptor-related enzyme activity." The transplasma membrane electron transport system could fullfill that role.

Acknowledgments

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