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Dependence of intestinal iron absorption on the valency state of iron

P. Wollenberg and W. Rummel

Institut für Pharmakologie and Toxikologie der Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

Summary. *1.* In rats iron was absorbed after administration into the gut lumen as ferric iron bound to serum albumin, to nitrilotriacetic acid, and to 8-OH-quinoline sulfonic acid, or as isolated diferri-transferrin. *2.* Iron absorption from ^{59}Fe-labelled transferrin was inhibited by the addition of rat plasma. *3.* The inhibitory component in the rat plasma turned out to be ceruloplasmin (ferrous iron oxidase, EC 1.16.2.1). *4.* The absorption of iron from these ferric iron complexes was also inhibited by addition to the incubation medium of ferrozine, a strong anionic Fe(II)-ligand. *5.* Uptake and absorptive utilization of transferrin-bound ferric iron was decreased after a prewash of the gut lumen and could be restored by the addition of ascorbate to the incubation medium. *6.* The conclusion was drawn from these results that luminal reduction precedes ferric iron absorption and that this is a prerequisite for the uptake into the mucosa.

Key words: Iron-absorption – Rat small intestine – Ascorbate – Ceruloplasmin – Reduction

Introduction

The question whether iron is absorbed from the small intestine exclusively in the ferrous form or also in the ferric form is not yet answered unequivocally. Although early reports were in favour of exclusive absorption of ferrous iron (Heubner 1926; Lintzel 1933; Moore et al. 1939; Venkatachalam et al. 1956) conflicting results were presented in more recent papers on this topic.

Huebers et al. (for reference see Forth and Rummel, 1973) described results, which showed that Fe(II) binding sites – with a high affinity for iron, but not for cobalt – exist only in brush borders from the jejunum. Marx and Aisen (1981) were able to demonstrate uptake of Fe(II) into an osmotically active space of rabbit intestinal brush border membrane vesicles whereas in their hands, no uptake of Fe(III) could be shown in the same preparation. Likewise, Muir et al. (1984) and Muir and Hopfer (1985) described ferrous iron binding sites and transport pathways preferentially localized in the upper part of the small intestine. The capacity of these Fe(II) binding sites in murine intestinal brush border membranes was dependent on the iron status of the animals. In contrast, Simpson et al. (1983, 1984, 1985) described ferric iron uptake into an osmotically active space of murine intestinal brush border membranes which they attributed to a saturable transmembrane transport process. When they repeated their experiments in vivo, however, they found transport rates which differed by a factor of ten from their earlier in vitro findings (Simpson and Peters 1986). Stremmel et al. (1987) report on an intrinsic protein in rat intestinal brush border membranes, which binds ferric and ferrous iron likewise and is considered to play a role in iron uptake.

Very often availability and absorption are not discriminated clear enough. Differences in absorption are not necessarily differences in absorption rate, i.e. the rate of uptake into the intestinal epithelium; they can also simply reflect differences in availability. The availability for absorption of Fe(II) is orders of magnitude higher than that of Fe(III) (Forth and Rummel 1973). This study is focussed exclusively on the dependence of iron uptake and transport by the intestinal mucosa to the body on the valency state of iron.

Changes in availability due to precipitation of ferric hydroxide may obscure the conditions of intestinal iron absorption (Lichtenberg 1966). In order to avoid this interference, iron(III)albumin-"complexes" and diferric transferrin were used as iron(III) sources in this study. Transferrin, the iron transporting protein of the plasma, occurs under in vivo conditions in the gut lumen (for references see: Huebers and Rummel, 1984) and in the apical area of duodenal villous cells (Banerjee et al. 1986) and is considered to play a part in iron absorption, particularly in iron deficient rats (Huebers et al. 1983b). Therefore iron-transferrin as a Fe(III)-complex of an extraordinarily high stability was included in the investigation.

If the uptake of iron into the mucosal cells is mediated by Fe(II) binding sites as proposed by Muir and Hopfer, then the reduction of Fe(III) iron should be an obligatory initial step in iron absorption. In order to support this hypothesis we tried to show that intestinal iron absorption is inhibited by *1.* counteraction of the reduction by an oxidase, *2.* removal of the reducing agent, and *3.* trapping of the reduced iron by a chelating agent.

Materials and methods

Chemicals. ^{59}Fe (0.074–0.148 TBq/g) was purchased as ferric chloride in 0.5 M HCl from New England Nuclear (Dreieich, FRG). Ferrozine, 8-hydroxyquinoline-5-sulfonic acid (8-HQS), and human ceruloplasmin (bufferred in 0.25 M NaCl – 0.05 M sodium acetate, 50–100 Curzon units/mg, 50 mg/ml) were from SIGMA (Deisenhofen, FRG). *Transferrin* was isolated from pooled plasma which

Send offprint requests to P. Wollenberg

was obtained from iron deficient donor rats by the following method: 25 ml of rat serum were saturated with iron and freed from albumin on a blue sepharose CL-6B column (Pharmacia, Freiburg, FRG) by the method of Travis and Pannell (1973). Fractions containing transferrin, as judged by absorbance at 465 nm, were pooled and concentrated to 25 ml in an Amicon cell equipped with an XM50 filter. The concentrate was applied to a column (100 cm long, 5 cm diameter) packed with sephacryl S-200 (Pharmacia, Freiburg, FRG) and eluted with 140 mM NaCl 5 mM phosphate buffer pH 7.4. The absorption of the eluate at 280 and 465 nm was measured and fractions with a ratio $E_{465}/E_{280} > 0.044$ were pooled and concentrated to a content of 10 mg/ml. The preparations were filtered through a sterile 0.2 µm-filter and kept on stock in sterilized vials at 4°C.

Rat *albumin* was recovered from the blue sepharose column by elution with 1.5 M KCl. It was dialyzed four times for twelve hours each against forty volumes of $(NH_4)_2CO_3$ (10 mM) and was lyophilized thereafter. All other chemicals were supplied by Merck (Darmstadt, FRG) and were from the highest analytical grade available.

Test-solutions. Radiolabelled rat serum transferrin was prepared according to Huebers et al. (1983a). The final solution contained 10 mg transferrin/ml 0.9% NaCl, 5 mM Na-phosphate pH 7.5. Fe-Albumin was prepared according to Huebers et al. (1983b) except that human albumin concentrate was replaced by purified rat albumin, which is free of contaminating transferrin. The final solution contained 10 µg Fe/ml at pH 7.5. Acidic iron solution contained 300 ngAt of Fe ($FeCl_3$) in 1 ml 0.001 *n* HCl, 0.9% NaCl.

The Fe-ferrozine complex (ferrozine = 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazin-(ar)-4′4″-disulfonic acid) was prepared by addition of 10 mM ascorbic acid solution (100 µl/ml) and 0.15 M ferrozine (10 µl/ml) to 1 ml of the acidic iron solution one hour before the onset of the experiment. The Fe-8-hydroxyquinoline-sulfonic acid complex was prepared by addition of 0.15 M 8-hydroxyquinoline-sulfonic acid (10 µl/ml) to 1 ml of the acidic iron solution one hour before the onset of the experiment.

When the respective ligands were used as "treatments", 10 µl of an 0.15 M solution was vortexed into one ml of the control solution immediately before it was injected into the isolated gut loop.

The sulfonated derivatives of 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazin and 8-OH-quinoline were chosen in order to guarantee an extraordinarily low absorbability of the iron chelates [for 8-hydroxyquinoline-sulfonic acid see Forth et al. (1974)].

The Fe-nitrilotriacetic acid complex was prepared according to Aisen et al. (1967).

Animals. Female Sprague Dawley rats (Charles River-Wiga, Sulzfeld, FRG) weighing 160 g were made iron deficient by repetitive phlebotomies and feeding an iron-free semisynthetic diet as described by Forth and Andres (1969) and were used when they had gained a weight of 190–210 g.

Absorption experiments. Animals were starved overnight before the onset of the experiment. Anesthesia was obtained by i.p. injection of ethylurethane solution (250 mg/ml, 4.5 ml/kg body weight). The animals were laparatomized and 20 cm of the upper jejunum, beginning at the ligament of Treitz were ligated. Test solutions (1 ml) were injected into these gut loops and the abdomen was closed with Michel clips. In some experiments, the luminal contents were removed by a perfusion of the gut loop with 10 ml of 38°C warm Ringer solution at a rate of 20 ml/min before the test solution was introduced into the loop (prewash). The animals were maintained for one hour in a thermostated chamber (38°C), the abdomen was reopened thereafter and the animals were exsanguinated by puncture of the abdominal aorta. The blood was removed by two perfusions of the vascular bed with 10 ml of warm saline. The exposed gut loop, liver, kidneys, and spleen were removed and counted for radioactivity. Since liver values proved to be representative for the iron content in all other tissues measured, only these values will be presented. The gut loop was opened and the residual luminal fluid was collected together with 20 ml of ice-cold buffered saline (0.9% NaCl, 1 mM phosphate pH 7.4), which was passed through the lumen.

Analytical methods. Radioactive iron was determined in a Packard Instruments series 9001 multichannel analyzer, set at a window from 0.86 to 1.2 MeV. A Packard Armac small animal whole body counter was used to determine the radioiron remaining in the carcass. The iron content was calculated after background correction, using a reference standard of known specific activity.

Iron was determined with a commercial kit (Merck, Darmstadt, FRG).

The o-dianisidine-oxidase activity of ceruloplasmin was quantified according to Schosinsky et al. (1974). Reducing components in the gut lumen were determined as ascorbate according to Omaye et al. (1979).

Statistical evaluation. Mean and standard error of the mean were calculated and differences between means were evaluated for significance by the *t*-test or by one way analysis of variance (ANOVA) and the Tukey test, whichever was appropriate (Weber 1980).

Results

In pilot experiments the absorption of iron from previously ^{59}Fe labelled transferrin in rat plasma was measured in tied off jejunal loops, prepared according to Ochsenfarth and Winne (1969), and found – unexpectedly – to be extraordinarily small. Even if diluted plasma (1:10) was added to isolated ^{59}Fe-labelled transferrin nearly no iron was absorbed. This result was in contrast to earlier findings, which showed, that diferric transferrin (di-Fe(III)-transferrin) is indeed a good source of iron for absorption (Huebers et al. 1983b).

The inhibitory principle in plasma could be attributed to a high molecular weight (protein) factor due to its lacking dialyzability. The only serum protein, which is known to play a part in iron metabolism besides transferrin is ceruloplasmin. Therefore purified ceruloplasmin was used in further experiments in order to determine, whether it might be responsible for the observed inhibition of iron absorption.

Since species differences seemed to be irrelevant in this context, commercially available human ceruloplasmin was used for the further investigations, the ferro-oxidase activity of which is higher than that of rat ceruloplasmin (Roeser et al. 1970).

In a dose equivalent to 1/10 the volume activity of rat serum (based on the diamino-oxidase-activity) ceruloplasmin was able to inhibit iron absorption about

Table 1. Effect of various treatment on the appearance of newly absorbed iron in the liver. Values are means ± SEM, an asterisk marks differences, which are significant with $p < 0.05$ versus respective control(s). (Abbreviations: 8-HQS = 8-hydroxy-quinoline-sulfonic acid, NTA = nitrilotriacetic acid)

Test solution	Treatment	Liver Iron (^{59}Fe) (ng)
^{59}Fe-transferrin	control	46.8 ± 6.8
^{59}Fe-transferrin	ceruloplasmin	9.1 ± 3.4*
^{59}Fe-albumin	control	250.0 ±33.1
^{59}Fe-albumin	ceruloplasmin	19.8 ± 3.3*
^{59}Fe-albumin	ceruloplasmin (boiled)	278.4 ±24.7
^{59}Fe-albumin	Cu^{2+}	248.1 ±22.4
$^{59}FeCl_3$ pH 2	control	314.6 ±52.0
^{59}Fe-ferrozine		12.6 ± 1.9*
^{59}Fe-ferrozine	8-HQS	16.9 ± 4.8*
^{59}Fe-8-HQS	control	228.4 ±48.7
^{59}Fe-8-HQS	ferrozine	34.2 ± 8.5*
^{59}Fe-NTA	control	6.52± 0.47
^{59}Fe-NTA	ferrozine	0.06± 0.04*
^{59}Fe-transferrin	control	41.5 ± 5.6
^{59}Fe-transferrin	washed	2.1 ± 0.2*
^{59}Fe-transferrin	washed + ascorbate	38.1 ± 3.3

95–100%. Therefore this dose was used in subsequent experiments.

When ceruloplasmin was given together with isolated ^{59}Fe-labelled di-Fe(III)-transferrin, absorptive utilization of iron was decreased from 46.8 ± 6.8 ng Fe in control animals to 9.1 ± 3.4 ng ($n = 6, p < 0.05$%, see Table 1) as represented by the ^{59}Fe content of the liver, which can be used as representative for total iron absorption (cf. Methods).

This inhibition (80%) is similar to that obtained by rat plasma dilutions having the same oxidase-activity, a result which confirms the hypothesis that ceruloplasmin is the factor responsible for the inhibition of iron absorption from whole rat plasma.

A similar, but even more pronounced inhibition was observed when ^{59}Fe-tagged albumin was used as the iron source (Table 1). The amount of absorbed iron appearing in the liver decreased from 250 ± 33 ng in controls to 20 ± 3 ng in the presence of ceruloplasmin. Simultaneously the luminal retention of iron increased from 2.41 ± 0.37 µg in controls to 4.16 ± 0.5 µg ($p < 0.05$) in the presence of the inhibitor. Neither boiled ceruloplasmin nor Cu^{2+} (given as $CuSO_4$) in amounts equivalent to the copper content of the ceruloplasmin were able to replace native ceruloplasmin.

The group treated with native ceruloplasmin was different from all others at a significance level of $p < 0.05$ in each group (statistics by ANOVA and Tukey-Test for simple contrasts), whereas no statistically significant differences were seen between controls and the animals treated with boiled ceruloplasmin or free inorganic copper.

When the influence of iron chelators on the absorptive utilization of iron administered as acidic Fe(III)-solution was investigated, it was seen that 8-OH-quinoline-sulfonic acid, which forms highly stable ferric iron complexes, does not inhibit iron absorption significantly (Table 1). In contrast, when using ferrozine, which forms highly stable complexes with ferrous iron, significantly less iron appeared in the liver, irrespective of the presence or absence of 8-OH-quinoline sulfonic acid in the test solution. Similarly, absorption of iron, if offered as an Fe-nitrilo-triacetic acid-complex, is inhibited by ferrozine (Table 1 d). The inhibition of iron absorption by ferrozine from the highly stable di-Fe(III)-transferrin is particularly pronounced. Values decrease from 43.5 ± 1.85 ng in controls to 1.3 ± 0.77 ng in the presence of the Fe(II)-chelator (Table 1).

If reduction precedes iron absorption, as indicated by the inhibitory action of ceruloplasmin, the question may be asked, whether a reducing agent is present in the luminal fluid or whether reduction is catalyzed by a reductase, which is localized in the brush border membrane. In order to answer this question, iron absorption from di-Fe(III)-transferrin was measured with and without previous perfusion of the gut lumen (Table 1). Previous washing decreased absorptive utilization from 41.5 ± 5.6 ng Fe in nonwashed controls to 2.1 ± 0.2 ng ($p < 0.05$). In order to quantify the reducing activity in the luminal fluid and assuming that ascorbate might be involved, the reducing activity was determined in an eluate by a method conventionally used for measuring ascorbate (Omaye et al. 1979). The eluate was obtained by rinsing 20 cm of gut with 0.5 ml saline. By this procedure, irrespective of the iron state of the animals, a total of 40–200 µg ($n = 6$) of ascorbate was obtained, which is a conservative estimate of the total amount present in the gut loop. This is in excess of the iron-dose used in the absorption experiments. After washing away the reducing agent it was tried to substitute it by ascorbate (1 mM) in the test solution. The absorptive utilization of iron was restored to control values as measured in prewashed animals, due to the addition of ascorbate (Table 1).

Discussion

The solubility of iron in its ferric form is extremely low in most physiological media except gastric juice. In this valency state, it is handled in chelated form by living organisms (Neilands 1981). Consequently, the transmembrane transport of iron requires either internalization of the unchanged complex by a carrier system, as it is the case in bacteria, or by endocytosis as in reticulocytes (Morgan 1974). Otherwise it requires previous reduction to yield the much more soluble ferrous iron, a mechanism by which iron is absorbed by plant roots (Sijmons et al. 1984) and, most likely, hepatocytes (Thorstensen and Romslo 1984; Morley et al. 1985). It can be hypothesized that either of these two mechanisms – or both – are operative also in the uptake of iron into the mucosal epithelium of the small intestine.

Uptake of ferrous iron into brush border membrane vesicles of normal and iron deficient mice has been shown by Muir and Hopfer (1985) to be higher in the proximal part of the small intestine than in the distal part. Furthermore, they were able to demonstrate an increased uptake of ferrous iron in iron deficiency only in vesicles derived from the proximal part of the organ. Thus, ferrous iron uptake in brush border membrane vesicles displays the same properties as iron absorption in vivo.

Results presented here show that reduction of ferric iron from various sources precedes iron absorption in the rat proximal small intestine, which is in very good agreement with the findings of Muir and Hopfer (1985).

In HE-LA-cells and hepatocytes, reductive internalization of iron is effected by a reductase which is located in

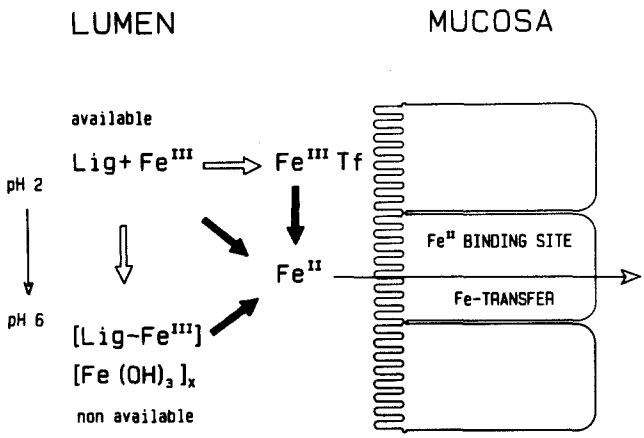

Fig. 1. Valency state and iron absorption. White arrows: binding of iron to ligands (Lig, Tf, OH^-, pH-dependent). *Black arrows:* valency change caused by luminal reducing agent(s). *Abbreviations: Lig*, ligands (e.g. phosphates), *Tf*, transferrin

the cellular membrane and which depends on intracellular NADH as an electron donor (Löw et al. 1986; Thorstensen and Romslo 1984; Morley et al. 1985). In contrast to this finding, reduction of ferric iron in the rat small intestine is dependent on luminal factors, which can be removed by a prewash of the gut segment. This became evident when the absorption of iron from transferrin complexes was compared in control vs. prewashed intestinal loops. A prewash abolished iron absorption from this highly stable ferric iron complex almost completely. Addition of ascorbate to the test dose completely reconstituted iron absorption in such loops. Therefore it can be concluded that di-Fe(III)-transferrin is not taken up by the gut epithelium unchanged, but must be cleaved reductively in the lumen.

Transferrin probably plays a role in trapping Fe(III) ionized by the gastric hydrochloric acid in the close neighborhood of the brush border membranes in the upper part of the small intestine (Huebers et al. 1974; Huebers and Rummel 1977). It keeps Fe(III) available for reduction and the subsequent binding and uptake into the epithelium. Thereby transferrin prevents the irreversible formation of polymeric ferric hydroxide, which is no more available for absorption. Simultaneously, due to its binding to mucus layer and/or the microvillous membrane (Huebers et al. 1975), transferrin would prevent a part of the iron to be moved together with the gut content, which is transferred relatively fast (within minutes) to more distal regions where iron absorption is less efficient (cf. Fig. 1). Additional pieces of evidence yielded the results obtained from ceruloplasmin experiments. It could be demonstrated, that ceruloplasmin inhibited absorption of Fe(III) iron completely. Obviously, due to its high affinity to Fe(II) and its oxidative capacity (Osaki et al. 1966) Fe(II) produced intermediarily by reducing agents was reoxidized efficiently; consequently, binding to the Fe(II) binding sites could not take place. This result is a strong argument in favour of the determining role of a reductive step for the initiation of iron absorption particularly from Fe(III) complexes with an extraordinarily high stability as e.g. di-Fe(III)-Tf.

One might speculate, whether ceruloplasmin entering the gut lumen together with the bile (Lorentz and Jaspers 1970) is able to modulate iron absorption, but when measuring the ceruloplasmin contents of bile in normal and iron deficient animals (data not shown), we found that these secretions are not different in the two groups and were not sufficient to account for a measurable inhibition of iron absorption at all. In other words, ceruloplasmin is merely a useful tool for proving the importance of reduction in the process of iron absorption, but its action, at least in the gut lumen, has no physiological meaning.

In summary it can be concluded, that a transport system for ferric iron is lacking in the brush border membrane of rat small intestine. A prerequisite for the uptake into the mucosa is the transformation to Fe(II). Responsible for the reduction of ferric iron are constituents of the luminal fluid; this (or these) reducing agent(s) is (are) able to liberate simultaneously iron from complexes as stable as diferric transferrin and 8-OH-quinoline. Consequently, ceruloplasmin, a ferrous iron oxidase, inhibits iron absorption when administered into the gut lumen, obviously by a reoxidation of Fe(II) to Fe(III) and thereby preventing its binding to the Fe(II) binding sites of the brush border membranes in the upper part of the small intestine.

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