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Pharmacology & Toxicology
ISSN 0901-9928

Involvement of Iron (Ferric) Reduction in the Iron Absorption Mechanism of a Trivalent Iron-Protein Complex (Iron Protein Succinylate)

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(Received February 24, 2000; Accepted May 9, 2000)

Abstract: Iron protein succinylate is a non-toxic therapeutic iron compound. We set out to characterise the structure of this compound and investigate the importance of digestion and intestinal reduction in determining absorption of the compound. The structure of the compound was investigated by variable temperature Mössbauer spectroscopy, molecular size determinations and kinetics of iron release by chelators. Intestinal uptake was determined with radioactive compound force fed to mice. Reduction of the compound was determined by *in vitro* incubation with intestinal fragments. The compound was found to contain only ferric iron, present as small particles including sizes below 10 nm. The iron was released rapidly to chelators. Digestion with trypsin reduced the molecular size of the compound. Intestinal absorption of the compound was inhibited by a ferrous chelator (ferrozine), indicating that reduction to ferrous iron may be important for absorption. The native compound was a poor substrate for duodenal reduction activity, but digestion with pepsin, followed by pancreatin, released soluble iron complexes with an increased reduction rate. We conclude that iron protein succinylate is absorbed by a mechanism involving digestion to release soluble, available ferric species which may be reduced at the mucosal surface to provide ferrous iron for membrane transport into enterocytes.

In the treatment of iron deficiency anaemia, most oral preparations are effective. Unfortunately orally administered iron compounds, and particularly iron sulphate, the oldest drug used in therapy, may cause irritation and damage to the intestinal mucosa (Laine *et al.* 1988, Hilman 1995).

Considerable effort has been devoted to ensuring better tolerability to efficacy for iron vehicles. Two types of polymeric ferric compounds have received particular attention, namely, ferric polysaccharides and iron protein succinylate. Ferric-polysaccharide complexes were extensively studied in the 1980's. It was suggested that breakdown of the compound in the intestinal lumen was important in determining the absorption rate (Johnson & Jacobs 1990). A positive therapeutic effect against iron deficiency anaemia without evoking gastrointestinal side effects has also been achieved through the synthesis of a trivalent iron complex of succinylated protein, ITF282 (Cremonesi *et al.* 1984). The compound is insoluble under the acid conditions present in the stomach, thus avoiding the release of large concentrations of potentially aggressive iron ions (Caramazza *et al.* 1990; Cremonesi & Caramazza 1993) into the small intestine. A recent comparative study of a ferric polysaccharide and iron protein succinylate suggested that both are well tolerated in children, but that iron protein succinylate was more effective in increasing iron stores (Haliotis & Papanastasiou, 1998).

The mechanism by which iron is taken up from the succinylated protein by the intestinal epithelial membranes has not yet been elucidated. By using ^{59}Fe as a tracer and monitoring the blood radioactivity, two iron succinylated proteins- ITF241 and ITF282 exhibited, in the rodent, similar pharmacokinetics profile and bioavailability as $FeSO_4$ (Simpson *et al.* 1991; Chasseaud *et al.*, personal communication). The iron uptake occurs mainly in the duodenum and gradually decreases along the intestine (Simpson *et al.* 1991). The mechanistic hypothesis of absorption is that, even though ITF282 is insoluble in the stomach (thus poorly available to absorption in this tract), iron may be released there and/ or the drug may be digested by proteases in the intestine, producing highly soluble iron succinyl peptides that are potentially able to release trivalent iron in a soluble form. On reaching the membrane, uptake of trivalent iron species (e.g. pinocytosis) or reduction of iron prior to absorption is envisaged to occur.

The mechanism by which trivalent iron is absorbed by the epithelial membrane is not clearly established, (Quian & Tang 1995). However, recent reports support the view that at the molecular level, reduction of iron to Fe^{2+} via a membrane ferri-reductase (Raja *et al.* 1992; Pountney *et al.* 1996) is an essential step for the iron absorption mode. A divalent

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cation transporter (DCT1) has been recently identified and cloned in the duodenal epithelial cells and characterised as a proton coupled metal ion transporter (Gunshin *et al.* 1997). The protein is able to transport, among other ions such as zinc, copper and magnesium, iron in the divalent state thus ensuring entry of the element into the cell by a non-receptor-mediated uptake system. Mutations in this protein lead to decreased absorption of dietary iron and both ferric and ferrous complexes (Fleming *et al.* 1998; Oates *et al.* 1996), implying that the principle iron absorption pathway is mediated by DCT1.

The system iron reductase – metal ion transporter could explain, at least in part, the absorption mode of trivalent iron derivatives, including those of a polymeric nature such as iron succinyl protein. This would require that the chemical and physical properties of the element (soluble at very low acid pH and insoluble at neutral/alkaline pH values) be reversed in order to permit the release of iron to the reductase in a medium (pH near 7) where normally iron is insoluble.

The present study was performed in order to investigate whether the mechanism of absorption of iron protein succinylate, as a prototype of structurally complex polymer iron donors, involves iron reduction. Studies on the iron absorption mechanism from derivatives containing iron in the trivalent form, that involve a reduction of Fe^{3+} have been conducted (Barrand *et al.* 1990), but no information is available on such mechanisms for polymeric (e.g. polysaccharides or protein matrices) iron complexes.

Material and Methods

Materials. Iron succinyl proteins: ITF241 (iron succinyl albumin) was labelled with ^{59}Fe as described previously (Simpson *et al.* 1991). The resulting labelled solution of ITF241 contained 16.8mM Fe with a specific activity of 37.9 nCi/nmol. ITF282 (iron succinyl casein) either in powder form (iron-succinyl protein containing 5% w/w Fe, Italfarmaco S.p.A. (Milan, Italy) or formulated in aqueous solutions (Ferplex®, 2.7% w/v, Italfarmaco S.A. Alcobendas-Madrid, Spain) was used as iron carrier derivative for structural, digestion, iron release and reduction studies.

Ferrozine, trypsin, pancreatin and pepsin were all obtained from Sigma Chemical Co. (Poole, Dorset, UK). Natural ferritins (horse spleen ferritin containing 27% iron), batho-phenanthroline, desferrioxamine, hydroxypyridone and thioglycollic acid were obtained from Aldrich Chemical Co. (Poole Dorset, UK). Analytical grade reagents were utilized for the preparation of buffer solutions.

ITF282 was prepared by reaction of $FeCl_3$ with succinyl casein according to Pat. No. 896.051 B. In brief: casein, dissolved in water, is extensively succinylated by reaction with excess of solid succinic anhydride at the constant pH value of 7.8 under controlled temperature conditions. The acylated protein is then purified from excess reagent (and free succinic acid) through repeated dissolution (at neutral pH) and precipitation (acid pH of 2.3). To 1 g of succinyl casein dissolved in H_2O a solution of freshly prepared $FeCl_3$, 0.5 g in 20 ml H_2O is added under controlled stirring. The pH decreases to 2.5 and the iron protein succinylate precipitates. Excess iron is removed by washing with 1 mM HCl and the derivative is dissolved at pH 7.5 by NaOH additions, dialysed and lyophilised.

Animals. Males, CD1, 6–8 week old mice were used throughout. Hypoxia was induced by placing mice in a hypobaric chamber for 3 days at 0.5 atmosphere (Raja *et al.* 1987). Iron deficiency was induced by feeding mice iron-free synthetic diet (Simpson 1996) for 3–4 weeks from weaning. Controls were fed an iron-supplemented diet (Simpson 1996). Purified low-iron diet had less than 1 mg Fe/kg and control diet was the same diet supplemented with 62 mg/ kg iron as $FeCl_3 6H_2O$. Food was given *ad libitum* except as indicated. Iron absorption studies were performed on mice fasted overnight in wire-bottom cages. The UK Home Office approved all animal procedures.

Iron succinyl protein absorption. Hypoxic mice were given 4.9 µg ^{59}Fe-succinyl-albumin in 50 µl of either 10 mM ferrozine or H_2O orally. The mice were left for 1 hr during which time they were offered H_2O or 0.5 mM ferrozine in H_2O as drinking water. In some experiments, two oral doses of 10 mM ferrozine or H_2O, as appropriate, were given at 20 min. and 40 min. after the ^{59}Fe dose. The mice were killed and the stomach, small intestine and caecum plus colon removed. The duodenum (5 cm) was washed out with 5 ml 0.15 M NaCl and the duodenal wall, washings, stomach plus contents, jejunum (5 cm), ileum, caecum and colon (all including contents) and carcass were counted for ^{59}Fe in a suitable gamma counter (LKB-Wallac 1282 Compugamma CS (Turku, Finland) or a twin crystal small animal whole body gamma counter). Hypoxic or normal mice were given 50 µl of a ^{59}Fe $(ferrozine)_3$ complex prepared by mixing $^{59}FeCl_3$, sodium ascorbate and ferrozine to give final concentrations of 50 µM, 1 mM and 0.5 mM respectively. Mice were killed after 1 hr and analysed for ^{59}Fe as above.

Mössbauer spectroscopy. Mössbauer spectra were obtained from ITF282 at temperatures from 77K down to 1.3 K. A conventional constant acceleration spectrometer was used with a ^{57}Co RH source and the spectra are plotted with the centre of the room temperature iron metal spectrum as the zero of velocity.

Release of iron by complexing agents. Experiments were carried out according to previously described methods (Cozzi *et al.* 1988). In non-reducing conditions, 2 mM desferrioxamine or hydroxypyridone were used as Fe^{3+} complexing agents. The absorbence variation at 425 (ε=2,770 M^{-1} cm^{-1}) or 460 nm (ε=4,600 M^{-1} cm^{-1}) were monitored as a function of time. Under reducing conditions, 2 mM batho-o- phenathroline was used as a Fe^{2+} complexing agent and 50 mM thioglycollic acid as the reducing agent. The reaction was monitored at 535 nm (ε=2,770 M^{-1} cm^{-1}).

Gel filtration of trypsin hydrolysed ITF282. Samples of ITF282 (Ferplex®) were digested with trypsin at constant pH (7.5 and at 37°) using a ratio equal to 0.01 w/w ITF282/Enzyme. The digest was chromatographed on a Sephacryl S-400 HR 16×65 column using 20 mM Tris and 150 mM NaCl pH 7.4 as eluant. Absorption was monitored at 301 nm. Polyacrylamide gel electrophoresis (PAGE) was also conducted on the digested ITF282.

Digestion and iron mobilisation. Ferplex® was digested *in vitro* with HCl and pepsin (Sigma Chemical Co., porcine pepsin A, 10 mg/ml) at 37° in a shaking water bath, then neutralized with solid $NaHCO_3$ and incubated with pancreatin (Sigma, porcine pancreas, 50 mg/ml). Studies of *in vitro* formation of Fe(II) and available Fe(III) by digests was performed by addition of supernatants from centrifuged (10,000×g, 5 min.) samples of digest to 0.2 M acetate buffer at pH 4.8, followed by addition of ferrozine (1 mM final) then ascorbate (1 mM final). Absorbance changes (562 nm), after addition of ferrozine or ascorbate, were recorded.

Iron reduction by mouse duodenum and ileum. The reduction experiments were performed using duodenal or ileal tissue isolated from mice. A piece of intestine was removed from the appropriate region and trimmed free of connective or pancreatic tissue. The piece was then cut longitudinally (so as to expose the mucosal surface) and cut into 2 or 3 fragments of roughly equal sizes. The fragments were

rinsed in 1 ml of physiological buffer (125 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 16 mM Na-Hepes, pH 7.4), blotted and pre-incubated in the same buffer (2 ml) prior to being transferred to 2 ml of the same buffer containing Ferplex® or its digest and ferrozine (1 mM). It is noteworthy that tissue from the same animal was used to ascertain reduction of either the Ferplex® or its digest: This procedure thus allows direct comparison of the reduction rates between the two. Aliquots (100 µl) were removed from ferrozine-containing media at appropriate time points (up to 15 min.) to establish a time-course for reduction. Changes in the optical densities (due to formation of the coloured Fe(II)-ferrozine complex) were corrected for basal values obtained by simultaneously performing incubations in the absence of tissue. The tissue was removed from the medium, blotted and weighed. Reduction values were corrected for tissue wet weight.

Results

Effect of ferrozine on absorption of iron succinyl protein.

Fig. 1 shows that ferrozine inhibits absorption of iron succinyl protein by hypoxic mice at the mucosal uptake step, diverting the iron to the distal small intestine. This is probably due to formation of the unabsorbed Fe(II)ferrozine complex. Hypoxic mice were used to induce specific duodenal iron absorption pathways, relative to non-specific pathways, such as paracellular absorption (Raja *et al.* 1987). Separate experiments demonstrated that absorption of ^{59}Fe(II)ferrozine complex is low (less than 10%) and shows no adaptive increase in hypoxic mice (data not shown), unlike absorption of iron succinyl proteins (Simpson *et al.* 1991).

Iron speciation and release.

Previous experimental analyses aimed at characterisation of ITF282 indicated that this iron donor is composed of an aquo-complex form of iron, bonded to succinyl-casein matrix. The iron was deduced to be a trivalent species by UV-VIS and ESR spectroscopy, complexation with desferrioxamine (Cozzi *et al.* 1988) and by the ferroxidase activity of casein and of succinylated casein (Arosio, unpublished data).

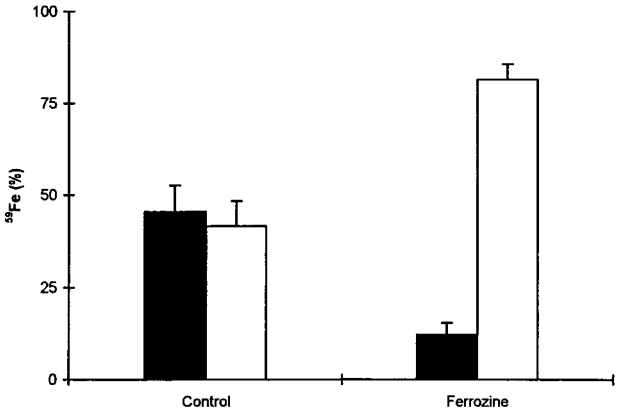

Fig. 1. Effect of ferrozine on absorption of iron succinyl protein. Mice (8 per group) were dosed with 4.9 µg ^{59}Fe-succinyl-albumin with or without (control) 10 mM ferrozine as described in Materials and Methods. Carcass ^{59}Fe (■) is expressed as a percentage of the radioiron which had left the stomach. Unabsorbed radioiron (□) was ^{59}Fe found in the whole intestinal tract, distal to the duodenum. Both carcass and unabsorbed ^{59}Fe values are significantly different from control ($P<0.001$).

The nature of the iron complex present in the ITF282 was investigated by gel filtration and Mössbauer spectroscopy in order to delineate the chemical characteristics of the iron derivative, especially in relation to the properties of iron release. Mössbauer spectroscopy provided more direct evidence on the iron species present in ITF282. Fig. 2 shows the Mössbauer spectra obtained at various temperatures between 77 and 1.3 K. The 77 K spectrum consists of a single quadrupole-split doublet, with a chemical shift of 0.50 mm/s, a quadrupole splitting of 0.74 mm/s and a relatively narrow linewidth of 0.61 mm/s. At temperatures below around 18 K the spectra also show evidence of a magnetically-split sextet component. This component progressively increases in intensity, at the expense of the doublet, as the temperature is lowered. In the lowest temperature spectra, obtained at 1.3 and 4.2 K, the sextet component dominates. However, the doublet component persists, even at 1.3 K and has parameters very similar to those of the 77 K spectrum. The sextet component has a magnetic hyperfine field of approximately 46 T at 4.2 K.

The nature of the matrix can affect the availability of iron and comparative evaluation was conducted between ITF282 and ferritin as structurally different polymeric iron proteins following exposure to complexing agents. Iron release from intact ITF282 measured as absorbance variation of ferrioxamine or Fe-(hydroxypyridone)$_3$ complex at different times in non-reducing conditions (a) or measured as Fe^{2+}-batho-o-phenanthroline chromogen in reducing conditions (b) is shown in fig. 3 (Corsi *et al.* 1994). The iron is rapidly released, reaching 50% of the total within 60 min. and tending then to a plateau. Hydroxypyridone shows the same profile as desferrioxamine but has higher mobilization capacity. Ferritin, at neutral pH values, presents a much lower release capacity compared with ITF282 both by reaction with the two trivalent iron chelators (desferrioxamine or hydroxypyridone) and by thioglycollic acid. Ferritin iron can be mobilised only at acid pH values and under reducing conditions (fig. 3b).

After treatment with proteases (trypsin as a model for pancreatin) the generation of peptides, that still contain iron, is evident from the gel filtration experiments shown in fig. 4. Starting from aggregated forms of ITF282 (fig. 4a) (mol. wt. 5,000 kD, composed of succinylated casein monomers, mainly α and β, mol. wt. 24,000 and 23,000 respectively as determined by PAGE), digestion produces smaller aggregates having apparent mol. wt. of 600 kD (composed mainly of succinyl casein fragments having molecular weight of 9–10 kD as determined by PAGE). The peaks eluting after 300 min. represented preservatives (methyl p-hydroxybenzoate and propyl p-hydroxybenzoate) used in the preparation of ITF282. These preservatives have strong absorption at 301 nm and were unaffected by digestive enzymes.

The ability of ITF282 protein and its related peptides

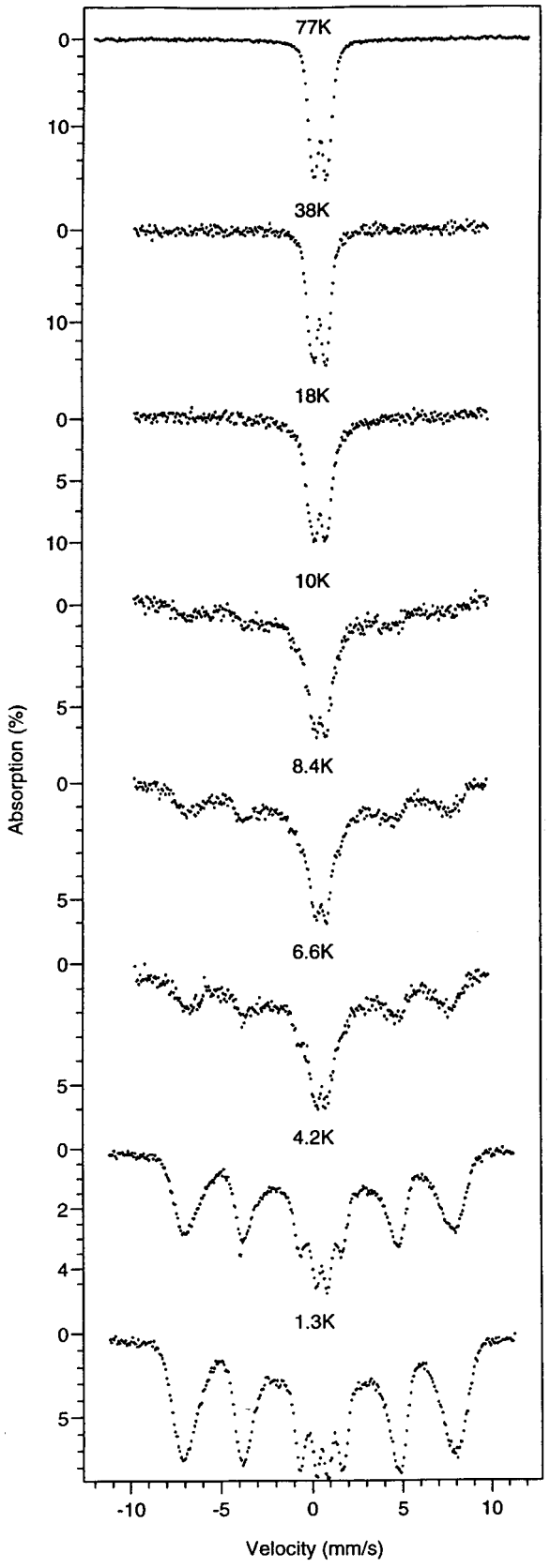

Fig. 2. Mössbauer spectra of ITF282. Spectra are plotted with the zero of velocity corresponding to the centre of the room temperature spectrum of iron metal.

(derived by the digestion step) to release iron and the nature of this released iron species was investigated by the ferrozine reaction. The reaction is able to distinguish the contemporary presence of divalent and trivalent iron species (Dorey *et al.* 1993), in acid conditions. In table 1 the availability of iron to ferrozine (Fe^{2+}) and to the ascorbic acid -ferrozine system (Fe^{3+}) is shown, after reaction with different amounts of pepsin and at different pH values. The combination of acid conditions comparable to those found in mouse stomach (pH 2.0) and pepsin was found to release significant quantities of iron (up to 15–20%). It can be observed that at acid pH, the release of iron in the divalent form is low and approximately 30 times less than the availability of trivalent iron. Pepsin at 10 or 50 mg/ml has little further effect in releasing iron from ITF282, with no significant increase in total Fe or Fe^{3+} released (table 1) over that obtained with acid alone. High pepsin concentrations (250 mg/ml) decreased iron release (table 1). This finding was unexpected and it is possible that high pepsin concentrations affected the solubility of released iron. Variation in the incubation time with pepsin up to 18 hr did not increase iron release at any pepsin concentration (not shown).

Digestion with pancreatin results in peptides that can release iron as shown in table 1. Little iron was released in the absence of the pancreatic enzymes. At 5 mg/ml pancreatin, iron release was significantly greater than untreated Ferplex® ($P<0.01$) or pepsin-treated and neutralised Ferplex® ($P<0.001$, table 1). Increasing concentrations of pancreatin (50 mg/ml) did not produce a significant further increase of the release effect at neutral pH values.

Reduction by mouse intestine.

Time dependent changes in Fe^{2+} formation for the incubation of ITF282 (Ferplex®) with duodenal tissue are shown in fig. 5. Changes in optical densities were small when incubations were performed in the absence of tissue and the data have been corrected for this basal level.

Time-dependent reduction of untreated ITF282 was observed on using tissue from normal CD1 animals. The calculated rate of reduction is about 28 pmol iron/min/mg tissue (fig. 5a, untreated Ferplex®). The reduction rate increases (65 pmol iron /min./mg tissue) in the experiments performed with digested Ferplex® (fig. 5a, 10 mg/ml pepsin followed by 50 mg/ml pancreatin-treated Ferplex®, $P<0.02$). Similar results were obtained for the groups fed iron-free synthetic diet, fig. 5b or synthetic diet supplemented with iron (not shown). Studies with digest prepared at lower pancreatin concentrations (5 mg/ml) showed little difference from the reduction rates seen with undigested Ferplex®, even at lower medium iron concentration (not shown).

The ileum was found to show some reduction of both Ferplex® and Ferplex® digested with 10 mg/ml pepsin and 50 mg/ml pancreatin. However, the data show lower reduction rates (fig. 5) compared to those obtained by duodenum for the digested sample, while reduction of undigested sample is similar. Also in these samples no increase in reduction in iron deficient, compared with iron replete mice was found.

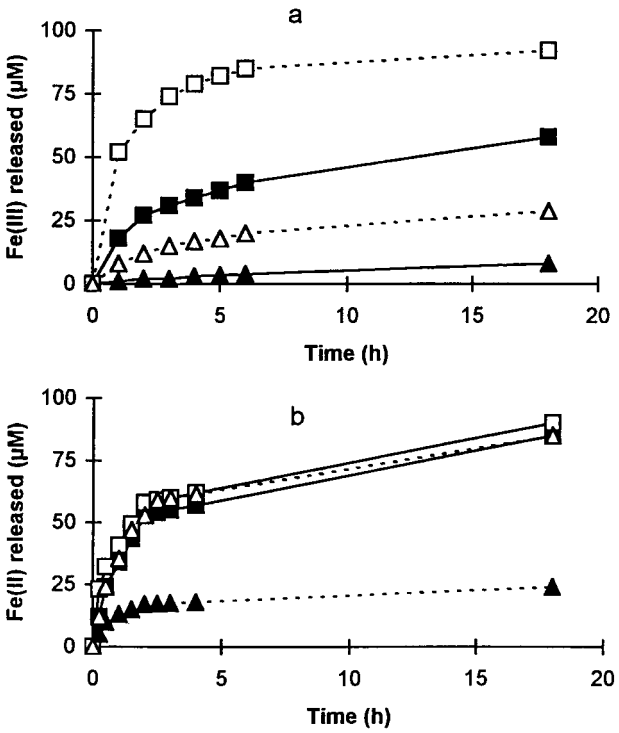

Fig. 3. Iron release from ferritin and ITF 282 *in vitro*. (a) 100 μM Fe as ITF 282 (■, □) or ferritin (Δ, ▲) were incubated with 2 mM desferrioxamine (■,▲) or hydroxypyridone (□, Δ) in 100 mM Hepes, pH 7.0. (b) As (a), only 2 mM bathophenanthroline disulfonate and 50 mM thioglycollic acid were included in place of ferric chelators. Incubations were carried out at pH 7.0 (100 mM Hepes (■, ▲)) or 5.5 (100 mM Na acetate (□, △)) using ITF 282 (■, □) or ferritin (△, ▲). Iron release was monitored spectrophotometrically as the appropriate Fe complex was formed.

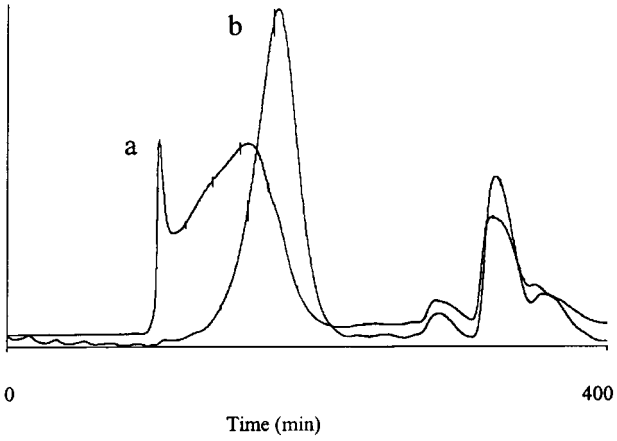

Fig. 4. Gel filtration of Ferplex® and digested Ferplex®. Ferplex® (a) and Ferplex® digested with trypsin (pH 7.5, 37°, 2 hr) (b) were separated on a Sephacryl S-400 HR column. Elution was monitored at 301 nm (y-axis). Calibration runs showed that proteins and peptides eluted before 300 min.

Discussion

The Mössbauer spectroscopy data support previous findings, which indicate that the iron in ITF282 is present in a trivalent state in the form of a complex structure assembled into small polymeric clusters. This valence state is indicated by the values of the chemical shift and quadrupole splitting at 77 K while the relatively narrow linewidth observed indicates that the iron site is essentially unique. The Fe^{3+} assignment is also consistent with the value for the magnetic hyperfine field of 46 T observed in the low temperature spectra. The progressive increase of the magnetic sextet fraction and concomitant decrease in the doublet fraction as the temperature is lowered is indicative of the phenomenon of superparamagnetism. This arises when a magnetically ordered material is present in the form of small particles (typically around 10 nm). The persistence of the doublet component at temperatures as low as 1.3 K suggests that the particle sizes go down to very low values ($<<10$ nm).

In vivo studies with radiolabelled iron succinyl protein

Table 1.

Release of Fe^{2+} or Fe^{3+} from Ferplex® (ITF282) by digestive enzymes.

Enzymes	mg/ml	pH	Fe^{2+} %	Fe^{3+} %	R	N
a)						
	–	7.3–7.5	0.08±0.02	0.98±0.14	19.2±8.6	6
	–	2*	0.29±0.05	10.72±3.48	29.1±7.9	9
Pepsin	10	2	1.15±0.22[z]	15.89±2.92	15.8±4.3	9
Pepsin	50	2	0.54±0.15	6.27±3.24	6.9±3.5[z]	8
Pepsin	250	2	0.66±0.08[z]	1.20±0.52[z]	1.8±0.8[z]	10
b) Pepsin, 10 mg/ml plus:						
Pancreatin	5	6.5–7.0	0.20±0.06[y]	3.01±0.71[xy]	17.8±8.8	10
Pancreatin	50	6.5–7.0	0.21±0.04[y]	4.00±1.94	17.4±8.7	4

Ferplex® was incubated in a shaking bath at 37° for 1 hr with the indicated pH and concentrations of pepsin. In a) Fe^{2+} or Fe^{3+} levels were determined as described in the methods. In b) Ferplex® was incubated with 10 mg/ml pepsin at pH 2 for 1 hr at 37°, then the pH was adjusted with solid $NaHCO_3$ and a further incubation in the presence of the indicated pancreatin concentration was conducted, with further determinations of Fe^{2+} or Fe^{3+} before the incubation and after 1 hr (only the latter values are shown). Values are expressed as % of the total iron (± SE). R=ratio of Fe^{3+} to Fe^{2+}. * Values between 1.38 and 2 were measured.

[x]$P<0.001$ versus before pancreatin incubation (paired t-test).

[y]$P<0.01$ versus untreated Ferplex® (pH 7.3–7.5).

[z]$P<0.05$ versus incubated at pH 2 without pepsin.

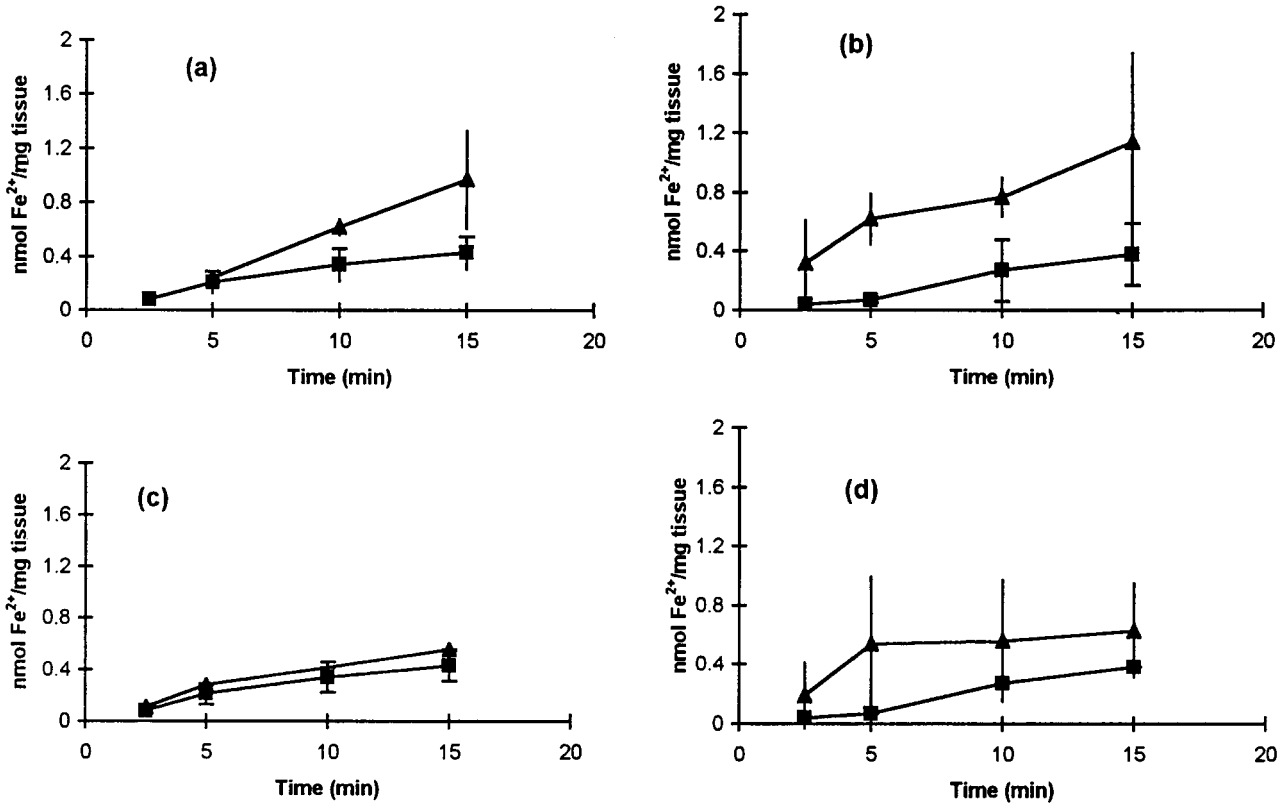

Fig. 5. Reduction of iron succinyl protein by mouse intestine. Mouse duodenal (a, b) or ileal (c, d) fragments were incubated with Ferplex® (■) or Ferplex® digested (37°) with 10 mg/ml pepsin for 1 hr at pH 2 then with 50 mg/ml pancreatin for 1 hr at pH 6.5–7.5 (▲). Fe^{2+} production was measured by formation of Fe(II)ferrozine complex at 562 nm. Mice were (a, c) normal CD-1 mice or (b, d) mice fed low iron diet.

show that a specific Fe^{2+} chelator, ferrozine, inhibits absorption of the iron, despite the fact that the original compound is ferric. The failure of ferrozine to completely inhibit iron absorption, down to levels seen with ^{59}Fe(II)ferrozine may be due to ferrozine toxicity in the gut or the presence of other pathways for iron absorption (Simpson *et al.* 1989; Conrad *et al.* 1994). It should be noted that the use of ferrozine in iron reduction experiments requires caution in interpreting the results as the rate and extent to which reactions occur may be different when ferrozine is not present (Marx 1995).

Solubilization and mobilization of iron are essential steps for iron absorption by mucosal cells (Bezkorovainy 1989). Different iron carriers may be expected to show different behaviour depending on the structure and accessibility of the element to the ligands (e.g. mucin, citrate, bile acids) present in the gastrointestinal juice or on the epithelial membrane. The solubility properties of ITF282 are such that it precipitates in the stomach, thus leading to its retention in that organ while digestive enzymes and acid can act to liberate soluble iron species (Simpson, unpublished observation). Table 1 provides support for this in showing significant release of iron in acid conditions in the presence of pepsin. The released iron species may then be solubilized by endogenous ligands (e.g. mucin, bile acids), dietary ligands (eg histidine, citrate) or digested ITF282 and made available for reduction and/or uptake in the proximal intestine.

The data shown strongly suggest that owing to its chemical characteristics and structure, iron can be mobilised from intact ITF282 by interaction with more stable complex-forming agents such as hydroxypyridone or desferrioxamine (fig. 3a) or by reduction at near neutral pH conditions (fig. 3b). This is indicative of an open structure with a high degree of accessibility. Digestion of the ITF282 with pepsin/pancreatin produces iron-containing peptides, endowed with high solubility, in a neutral pH medium, and constituted by small iron succinyl-casein fragments from which iron can be mobilized or made available.

Two iron species, bi- and trivalent could be determined, the trivalent one being prevalent (65–98% of iron). Pepsin alone shows a poor capacity for digesting ITF282 as no marked increase in total iron release could be found. Pancreatin digestion does lead to increased iron release. The most favourable conditions (10 mg/ml pepsin and 50 mg/ml pancreatin, both incubated for 1 hr) released 4% of iron, 95% of it as Fe^{3+}. The data suggest that ITF282 protein itself has little capacity to reduce Fe^{3+}, even after digestion. Casein has a low content of cysteine (Croft 1980). The reduction of Fe^{3+} to Fe^{2+}, necessary for absorption through DCT-1, must therefore be mediated by reductants supplied

by digestive secretions (Wien & Van Campen 1991) or by mucosal reductase activity (Raja *et al.* 1992; Pountney *et al.* 1996). Increased solubility of the peptides that still contain iron, at neutral /alkaline pH values, would permit the reaction with the iron reductase activity present in the intestine. The present results give evidence to support this hypothesis of digestion of the compound followed by iron release and reduction as a step for absorption of iron from ITF282. The experiments conducted here do not distinguish between mucosal reductase and release of reducing agents such as ascorbate or thiol containing metabolites (Wien & Van Campen 1991).

In the mouse a higher reduction rate by the duodenum was observed for the digested Ferplex® compared to the untreated Ferplex® (see fig. 5a). This behaviour is maintained in the reaction with duodenal tissue from mice fed with an iron-free diet fig. 5b, although no absolute increase in reduction rate was observed. Such an increase might be expected as a consequence of the increased expression of inducible reductase (Raja *et al.* 1992; Pountney *et al.* 1996), and of the increased absorption of the iron in the severe model of the anaemic rat (Pagella *et al.* 1984). This could be explained by the fact that the rate of reduction is regulated, in the presence of iron peptides as substrates, by the diffusion of the peptide to the reaction site of the enzyme. In the case of these *in vitro* experiments, ITF282 peptides produced by digestion still have molecular weights that can affect the diffusion. *In vivo* association with some other intestinal factors (mucins, integrins) cannot be eliminated as a cofactor affecting the diffusion of peptides towards the active site of the reductase. Alternatively, unregulated release of reducing factors by the tissue may explain the reduction.

By using jejunum/ileum, very low reaction rates were observed. Interestingly, there is no differentiation between the rate of reduction of untreated or digested ITF282. This is consistent with the poor iron reductase activity in this part of the intestine and, at the same time this datum indicates that the reduction of iron from ITF282 and related peptides observed with the duodenum is actually related to an iron reductase activity.

At present it is not possible to confirm that this is the only mechanism of iron absorption from ITF282 (or from other structurally similar trivalent polymeric iron donors). However, the availability of soluble trivalent iron species derived from digestion of ITF282 to duodenal reductase leads to divalent iron useful for the membrane uptake and internalization that occurs *in vivo*.

Acknowledgements

This is a contribution from the King's College Centre for the Study of Metals in Biology and Medicine. The work was supported by Italfarmaco SA.

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