Comparative study of iron mobilization from haemosiderin, ferritin and iron(III) precipitates by chelators

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The heteroaromatic chelators 1,2-dimethyl-3-hydroxypyrid-4-one, maltol, mimosine and 2,4-dihydroxypyridine-*N*-oxide, have been shown to mobilize iron from human spleen haemosiderin, ferritin and also from iron(III) precipitates, all containing equal amounts of iron, at physiological pH. In the case of almost every chelator, the least-solubilized polynuclear iron form was ferritin, whereas haemosiderin was more soluble and the iron(III) precipitate the most soluble of all. Most of the chelators were more efficient than desferrioxamine at releasing iron from ferritin, but less efficient in the removal of iron from the other two polynuclear iron forms. It is suggested that the chelator differences in iron mobilization may be related to variations in the chelator molecular structure, the protein structure, iron forms and in the mechanism of iron release.

INTRODUCTION

Under normal conditions iron is stored in a polynuclear form, mainly as ferritin and also as haemosiderin (Finch & Finch, 1955). In transfusion-dependent thalassaemia and other diseases of iron overload, excess iron, mainly in the form of haemosiderin, accumulates in the liver, spleen, heart and other tissues, causing damage (Finch & Finch, 1955; Iancu *et al.*, 1977). Similarly, accumulation of iron in the synovial fluid of rheumatoid-arthritis patients (Muirden, 1966) may also be associated with damage to this tissue. It has been suggested that haemosiderin itself may be related to the pathogenesis of tissue damage observed in iron overload by causing lysosomal disruption (Peters *et al.*, 1977), but other forms of iron toxicity, such as that caused by oxygen-activated products which are generated from iron catalysis, may also be involved (Wilson, 1977; Heys & Dormandy, 1981; O'Connell *et al.*, 1985).

Ferritin is a water-soluble, hollow, spherical-shaped protein of M_r 450000. It contains 24 polypeptide chains of two differing- M_r monomers (21000 and 19000) (Drysdale, 1977) and variable iron content (0–4500 iron atoms). Iron transport in and out of the protein is thought to be mediated through channels (Rice *et al.*, 1983). Several chelators have been shown to mobilize iron from ferritin, and the amount of iron released seemed to depend on conditions such as the molecular structure of the chelators, the presence of reducing agents, etc. (Pape *et al.*, 1968; Dognin & Crichton, 1975; Crichton *et al.*, 1980; Tufano *et al.*, 1981; Kontoghiorghes, 1986a). Haemosiderin is a non-water-soluble variable mixture of iron, other metals, protein and lipid fractions (McKay & Fineberg, 1964; Weir *et al.*, 1984). The origin of haemosiderin is not certain, but it may be formed from the proteolysis (Fischbach *et al.*, 1971) or polymerization of ferritin (Sturgeon & Shoden, 1964; Hoy & Jacobs, 1981).

The presence of an equilibrium between polynuclear, oligonuclear and mononuclear iron forms, which are related to the iron-solubility constant, the pH, the presence of chelating molecules and other factors (Schubert, 1964; Pitt & Gupta, 1975; Kontoghiorghes, 1982), make the assessment of the source of iron mobilization and of toxicity difficult. It has been suggested that the catalytic formation of oxygen-activated products by 'free' iron may play a role in the tissue damage observed in iron overload and many other diseases (Halliwell & Gutteridge, 1984; Slater, 1984; Editorial, 1985). The pathology of iron overload, however, is very complicated (Hoffbrand, 1980; Jacobs, 1980), and in many diseases the presence of excessive quantities of iron of any form in a tissue could be a continuous hazard and a source of toxicity unless it is removed.

The treatment of iron overload with subcutaneous desferrioxamine (DF), which is administered at a dose of 50–60 mg/kg over 8–10 h/day using a pump, prolongs the life of regularly transfused thalassaemia patients. This chelator, however, is expensive to produce and is orally inactive. Some new α -oxohydroxy chelators were shown to mobilize iron from transferrin *in vitro* (Kontoghiorghes, 1986b; Kontoghiorghes & Evans, 1985) and also in mice and rabbits when administered either intragastrically or intraperitoneally (Kontoghiorghes, 1982, 1985). These chelators and DF have also been shown to mobilize iron from horse spleen ferritin in a slow reaction process (Kontoghiorghes, 1986a). In the present work the mobilization of iron from human spleen ferritin, haemosiderin and FeCl₃ precipitate, all of equal iron content, was studied by using DF and other chelators of different structure and charge in order to examine whether a possible relationship exists between chelator structure and iron solubilization from these three polynuclear iron forms.

Abbreviations used: PBS, phosphate-buffered saline (138 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.3); 2 × PBS, double-strength PBS; L₁, 1,2-dimethyl-3-hydroxypyrid-4-one; L₂, 2,4-dihydroxypyridine-*N*-oxide; DF, desferrioxamine; NTA, nitrilotriacetic acid.

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EXPERIMENTAL PROCEDURES

Materials

1,2-Dimethyl-3-hydroxypyrid-4-one (L_1) was prepared as previously described (Kontoghiorghes, 1982) by a method based on that used for the preparation of other pyridone derivatives (Harris, 1976). [For different methods of preparation of L_1 , see also Yasue *et al.* (1970) and Severin & Loidl (1976).] 2,4-Dihydroxypyridine-*N*-oxide (L_3) was prepared as previously described (Kontoghiorghes, 1982) by the acid hydrolysis (20% HCl, 105 °C, 13 h) of 2,4-dimethoxypyridine-*N*-oxide (Talík, 1961). Maltol was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.; DF was from Ciba-Geigy, Horsham, Sussex, U.K.; PBS was from Oxoid, Basingstoke, Hants., U.K.; mimosine and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were from Sigma, Poole, Dorset, U.K.

Isolation of haemosiderin and ferritin

Ferritin and haemosiderin were prepared by using a method similar to those of McKay & Fineberg (1964) and Weir *et al.* (1984). A spleen obtained after splenectomy of an iron-overloaded thalassaemia patient was washed six times with PBS at 4 °C and stored at -20 °C. A piece of spleen (200 g) was cut into smaller pieces, washed twice with distilled water and homogenized in stages in NaCl (800 ml, 0.15 M). The homogenate was filtered through gauze and the filtrate centrifuged at 5000 *g* for 30 min. The supernatants were retained for ferritin preparation and the haemosiderin-containing pellets were washed by resuspending them in NaCl (500 ml, 0.15 M) and centrifuging at 5000 *g* for 30 min. The pellets were then suspended in KI (480 ml, 4.1 M), centrifuged at 70000 *g* for 2 h, pooled, freeze-dried and ground to a fine reddish-brown powder.

For the ferritin preparation the supernatants (see above) were pooled and heated to 70–80 °C for 10 min. The pooled solution was allowed to cool to 20 °C, the pH was adjusted to 4.7 with acetic acid and the suspension was left for 12 h at 4 °C. After this period the suspension was centrifuged (2500 *g*, 30 min) and the precipitate discarded. The resultant supernatant was saturated to 50% with $(\text{NH}_4)_2\text{SO}_4$, kept for 48 h at 4 °C, centrifuged at 2500 *g* for 20 min and the brown ferritin-containing pellet redissolved in distilled water. The ferritin solution was then passed through a Sephadex G-200 column, equilibrated with PBS, and stored at 4 °C in the same buffer for later use.

Study of the reactions of the chelators with polynuclear iron forms

The amount of iron present in the ferritin and haemosiderin preparations and a fresh aqueous FeCl_3 solution

Fig. 1. Polynuclear iron mobilization using high concentrations of chelators and iron

The reactions of L_1 , L_3 , maltol, mimosine and DF (12 ml, 4×10^{-3} M, pH 7.45 in $2 \times$ PBS) at 37 °C with ferritin (■), haemosiderin (▲) and a FeCl_3 precipitate (●), all containing 1.3 mg of iron, enclosed in dialysis tubing (2 ml) were studied by measuring the change in absorbance with time of the chelator-iron complexes in the diffusates at 460, 400, 420, 460 and 430 nm respectively. The arrow in the reaction with mimosine indicates breaking of the dialysis tubing (see legend to Table 1).

Fig. 2. Study of polynuclear iron mobilization by chelators using triplicate incubations

The reactions of L₁, L₃, mimosine and DF (12 ml, 1×10^{-3} M, pH 7.45 in $2 \times$ PBS) at 37 °C with ferritin (■), haemosiderin (▲) and a FeCl₃ precipitate (●), all containing 337 μg of iron, enclosed in dialysis tubing (2 ml) were studied in three separate experiments, by

was determined by atomic-absorption spectrophotometry after acid (HNO₃) digestion [3 ml (11 M)/10 ml of solution] and deionized-water dilutions. The concentration of the isolated ferritin was estimated as 1.67 mg/ml by using the Lowry *et al.* (1951) method of protein determination (bovine serum albumin was used as the standard). The mean iron content of the ferritin solution was determined to be 3246 molecules of iron per ferritin molecule. Two experiments using different chelator and iron concentrations were performed. In the first experiment, dialysis tubing containing ferritin (1.3 mg of iron/2 ml), haemosiderin solid [1.3 mg of iron (7.49% iron, w/w) in 2 ml of $2 \times$ PBS] and FeCl₃ (0.2 ml, 1.3 mg of iron) precipitate, which was formed after the addition of 1.8 ml of $2 \times$ PBS to aq. FeCl₃ and 20 min incubation, were all immersed in a chelator (12 ml, 4 mM) solution in $2 \times$ PBS (pH 7.45) and stirred continuously at 37 °C in stoppered glass tubes. In the second experiment, which was performed in triplicate under the same conditions, the chelator concentration in the glass tubes was 1 mM and the iron content in the dialysis tubing was 337 μg. During the reactions the absorption spectra of the chelator-iron complexes in the diffusates were recorded and, at the end of the reaction, the amounts of iron mobilized were estimated by atomic-absorption spectrophotometry and also from their absorption coefficients. Changes in the pH of the reactive mixtures were also measured at the end of the reactions.

RESULTS

General observations of the reaction of the chelators with polynuclear iron forms

The mobilization of iron by chelators from haemosiderin, ferritin and FeCl₃ precipitates is shown in Figs. 1 and 2 and Tables 1 and 2. Whereas most of the reactions reached completion in the incubations of the first experiment (Fig. 1) this was less evident with haemosiderin and ferritin under the conditions of the second experiment, where iron mobilization seemed to continue. In the second experiment, larger quantities of iron were mobilized more quickly in all the incubations of the chelators with FeCl₃ precipitates in contrast with their incubations with either ferritin or haemosiderin (Fig. 2). The amounts of iron mobilized by the chelators from the three different iron forms estimated by atomic-absorption and visible spectrophotometry were, in decreasing order: iron(III) precipitate > haemosiderin > ferritin (Table 2). The amounts of iron released during the first experiment were much higher than during the second experiment, where lower chelator and polynuclear iron concentrations were used (Tables 1 and 2). A decrease in the pH (mainly in the first experiment) of the chelator diffusates was observed after their reactions with haemosiderin and the FeCl₃ precipitate (Table 1).

The solubilization reaction of the FeCl₃ precipitate by chelators reached equilibrium at 1–3 days (Figs. 1 and 2); although in some cases the amount of iron released was

measuring the change in absorbance with time, of the chelator-iron complexes in the diffusates at 460, 400, 460 and 430 nm respectively. The bar at each point represents the s.d. of the mean for three experiments. The mean amount of iron (μg) mobilized ($\bar{x} \pm$ s.d.) at the end of the reaction is also shown.

Table 1. Mobilization of iron from polynuclear iron forms using high concentrations of chelators

Ferritin (1.3 mg of Fe), haemosiderin (1.3 mg of Fe) and an FeCl_3 precipitate (1.3 mg of Fe) enclosed in dialysis tubing (2 ml) were dialysed by continuous stirring at 37 °C against a chelator solution (12 ml, $\sim 4 \times 10^{-3}$ M, pH 7.45 in $2 \times \text{PBS}$). After 9.4 days the amount of iron mobilized was estimated by atomic absorption (six dilutions) and visible spectrophotometry, by using the following absorption coefficients ($\text{M}^{-1} \cdot \text{cm}^{-1}$): L_1 -iron complex, ϵ_{460} 4600; L_3 -iron complex, ϵ_{400} 5400; DF-iron complex, ϵ_{400} 2372; maltol-iron complex, ϵ_{420} 3720; mimosine-iron complex, ϵ_{460} 4654.

Iron form	Chelator (3.43×10^{-3} M)	pH of dialysates	Iron released (μg)	
			Atomic absorption ($n = 6$, $x \pm \text{s.d.}$)	Visible spectra
Ferritin	L_1	7.5	508 ± 45	528
	L_3	7.5	674 ± 16	690
	DF	7.3	418 ± 16	482
	Mimosine	7.3	$1247 \pm 98^*$	612
	Maltol	7.3	246 ± 22	366
Haemosiderin	L_1	6.8	778 ± 23	794
	L_3	7.0	612 ± 16	647
	DF	6.8	1127 ± 31	1228
	Mimosine	7.0	789 ± 26	819
	Maltol	7.0	332 ± 12	366
FeCl_3 precipitate	L_1	6.8	807 ± 23	794
	L_3	7.0	724 ± 16	725
	DF	6.8	1292 ± 35	1347
	Mimosine	7.0	720 ± 19	819
	Maltol	7.0	536 ± 21	560

* In the reaction of ferritin with mimosine, the dialysis tubing was damaged and the amount of iron was estimated spectrophotometrically by using a previous method (Kontoghiorghes, 1986a).

Table 2. Mobilization of iron from polynuclear iron forms using triplicate incubations

Ferritin (337 μg of Fe), haemosiderin (337 μg of Fe) and an FeCl_3 precipitate (337 μg of Fe) enclosed in dialysis tubing (2 ml) were dialysed by continuous stirring at 37 °C against a chelator solution (12 ml, $\sim 1 \times 10^{-3}$ M, pH 7.45 in $2 \times \text{PBS}$) in triplicate. After 7.8 days the amount of iron mobilized was estimated by atomic-absorption and visible spectrophotometry, the following absorption coefficients being used ($\text{M}^{-1} \cdot \text{cm}^{-1}$): L_1 -iron complex, ϵ_{460} 4600; L_3 -iron complex, ϵ_{400} 5400; DF-iron complex, ϵ_{400} 2372; mimosine-iron complex, ϵ_{460} 4654.

Iron form	Chelator (8.57×10^{-4} M)	Mean pH ($n = 3$)	Iron released (μg)	
			Atomic absorption ($n = 3$, $x \pm \text{s.d.}$)	Visible spectra ($n = 3$, $x \pm \text{s.d.}$)
Ferritin	L_1	7.3	84 ± 10	83 ± 7
	L_3	7.3	83 ± 6	80 ± 5
	DF	7.3	64 ± 9	61 ± 8
	Mimosine	7.3	73 ± 10	70 ± 19
Haemosiderin	L_1	7.3	124 ± 10	125 ± 12
	L_3	7.3	102 ± 13	94 ± 15
	DF	7.2	168 ± 26	155 ± 26
	Mimosine	7.3	99 ± 7	93 ± 6
FeCl_3 precipitate	L_1	7.1	194 ± 6	203 ± 5
	L_3	7.2	169 ± 7	173 ± 1
	DF	7.1	379 ± 32	352 ± 17
	Mimosine	7.1	173 ± 7	168 ± 6

similar to that from haemosiderin. In the latter case longer incubation periods (up to 6 days) were needed for the reaction to reach completion (Fig. 1).

Effect of each chelator on iron mobilization

DF caused the release of the largest amounts of iron from haemosiderin and from the FeCl_3 precipitates in

comparison with other chelators. Under the same conditions, however, it caused the release of only about 20–30% of ferritin iron, which, with the exception of maltol, was lower than that achieved by the other chelators, especially L_1 and L_3 . These two chelators (L_1 and L_3) were also effective in the mobilization of the largest amounts of iron from haemosiderin and the

iron(III) precipitates, in comparison with the other bidentate chelators (Tables 1 and 2).

Of all the chelators studied, maltol caused the smallest amounts of iron to be released from the three polynuclear iron forms. In the first experiment mimosine caused the mobilization of equivalent amounts of iron from both the iron(III) precipitate and haemosiderin, but in the former, equilibration was observed in 2 days, whereas in the latter it was observed in 6 days (Table 1). Ferritin iron mobilization was slower, and smaller amounts of iron were released than from the other two polynuclear forms (Figs. 1 and 2). The overall effects of mimosine, however, were comparable with those of L_1 and L_3 (Tables 1 and 2).

DISCUSSION

In this comparative study, differences were observed between the mode of action of the chelators and the iron-solubility properties of ferritin, haemosiderin and a $FeCl_3$ precipitate. In general, the mobilization of iron from all polynuclear iron forms was slow, taking more than a day to reach completion. The observation that all the chelators cause faster iron mobilization with the freshly prepared $FeCl_3$ precipitate compared with that with ferritin and haemosiderin may be related to the presence of either more soluble iron fractions, such as mononuclear or oligonuclear iron forms, in the $FeCl_3$ precipitate or to the different solubilities and structural compositions of the micellar iron forms present in these three preparations (Fischbach *et al.*, 1971; Harrison *et al.*, 1974; Massover & Cowley, 1975; Gray, 1975). Similar results were observed in the reductive mobilization of freshly added radioactive iron to iron-containing ferritin by Hoy *et al.* (1974). However, in those studies the reductive mobilization of the same amount of radioactive iron incubated for longer periods with ferritin was almost identical with that of ferritin itself. Other possibilities may exist, however, in that different micellar iron forms may be formed *in vitro* and *in vivo* and have different solubilities, and also that freshly formed iron precipitates are more soluble than older ones (Sturgeon & Shoden, 1964; Fischbach *et al.*, 1971; Richter, 1978). Furthermore, the concentration of the chelators and iron will also effect the overall amount of iron released (Tables 1 and 2).

The amounts of iron mobilized by all the bidentate chelators, except maltol, from the $FeCl_3$ precipitate is almost equivalent to the full chelating capacity of the chelator which is 3 mol of chelator/mol of iron. Maltol was the least effective chelator in the mobilization of all three polynuclear iron forms. The limited effectiveness of maltol in this and other experimental systems of iron mobilization (Kontoghiorghe, 1982, 1986a) decreases the prospects of its use in iron removal, but by no means the prospects of its use as an iron donor (Kontoghiorghe, 1982). DF, which is a hexadentate chelator and has greater capacity for chelation of 1 mol/mol of iron, caused the complete solubilization of the $FeCl_3$ precipitate and of most haemosiderin iron after prolonged incubations. The comparative inefficiency observed in ferritin iron mobilization by almost all the chelators, and DF in particular, may be related to the kinetic restrictions imposed by the ferritin structure, in which the presence of channels limits free access of large chelating molecules such as DF to the iron core and slows down the diffusion

of their iron complexes out of ferritin and also to the presence of amino acids in ferritin with chelating sites that compete with the chelators for iron.

An additional property other than the size of a chelator, namely its charge, may also be important in iron mobilization from ferritin at physiological pH. Positively charged molecules, such as DF, may be less effective than neutral chelators such as L_1 or negatively charged chelators such as L_3 or zwitterionic chelators such as mimosine. Increased mobilization of ferritin iron by analogues of L_3 (Kontoghiorghe, 1982) and NTA (Pape *et al.*, 1968), which are negatively charged, was also shown previously.

The faster solubilization of haemosiderin compared with ferritin iron in almost all chelator incubations suggests that the haemosiderin iron pool is more labile than that of ferritin (Fig. 2). Although this difference may be related to the isolation and reaction conditions *in vitro*, haemosiderin iron mobilization is also easily demonstrated *in vivo* after intensive treatment of thalassaemia patients with DF (Hoffbrand, 1980; Cohen *et al.*, 1985).

The decrease in the pH of some diffusates, which is associated with iron binding, is related to the H^+ release from the hydroxy group of the chelating sites of all the chelators during iron binding. The decrease in pH is less evident in the incubations with ferritin because of the buffer capacity of the protein shell of this molecule.

The present results suggest that the high efficiency of L_1 and L_3 in the mobilization of polynuclear iron forms and their oral effectiveness in removing iron *in vivo* (Kontoghiorghe, 1985; Kontoghiorghe & Hoffbrand, 1986) increases their (or their analogues; Kontoghiorghe, 1982, 1986c) prospects for clinical use in the treatment of iron overload and other forms of iron imbalance and also the use of chelators as probes in iron metabolic studies.

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