Decrease Solubilisation of Ferritin Iron and Fresh Iron(III) Precipitate Following Repeated Chelator Treatments

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Abstract

Iron solubilisation following repeated, long term chelation of horse spleen ferritin iron and an iron(III) precipitate at physiological pH has been examined spectrophotometrically using 1,2-dimethyl-3-hydroxypyrid-4-one (L_1)*, 1-methyl-3-hydroxypyrid-2-one (L_2)* maltol and desferrioxamine (DF). Iron solubilisation of human spleen ferritin by the chelators 1,2-dimethyl-3-hydroxypyrid-4-one, desferrioxamine, mimosine and 1,4-dihydroxypyrid-2-one (L_3)* was also studied using atomic absorption spectrophotometry. The chelators 1,2-dimethyl-3-hydroxypyrid-4-one and 1,4-dihydroxypyrid-2-one caused the highest amount of iron mobilisation from ferritin. When the chelator-treated ferritin and iron(III) precipitate were remixed with a fresh chelator solution of the same concentration, iron solubilisation decreased substantially in all the cases. It is suggested that the decrease in the mobilisation of iron by chelators observed following repeated incubations, is due to the decrease of the solubilisation of the remaining iron core and this may be related to the observed decrease in iron excretion of β-thalassaemia patients treated with repeated subcutaneous administrations of desferrioxamine.

Introduction

Iron is essential for normal growth and development. It is stored intracellularly mainly in a polynuclear form as ferritin or haemosiderin, the latter predominating in conditions of iron overload [1]. Ferritin is a water soluble hollow, spherical shaped protein of Mr of 450 000 which can store 0–4500 molecules of iron in a crystalline form [2]. Iron is thought to move in and out of the core through channels [3].

Iron mobilisation from ferritin at physiological pH is of great importance to those designing iron chelators for the treatment of iron overload in thalassaemia and other diseases of iron imbalance [4]. Desferrioxamine is an expensive iron chelator which is used subcutaneously for the treatment of transfusional iron overload in thalassaemia. This chelator is not effective in the mobilisation of iron from transferrin [5, 6] but it can mobilise iron from ferritin [7, 8]. Several new *N*-hydroxypyrid-2-one chelators e.g.. L_3 and α-ketohydroxypyridine chelators e.g. L_1, were shown to be effective in the mobilisation of iron from transferrin at physiological pH [6] and also from iron loaded animals when administered orally or parenterally [4, 9–11]. Some of these chelators and DF were also shown to mobilise iron from horse spleen ferritin in a slow reaction taking days to reach completion [12].

The long term repeated administration of DF to thalassaemia patients could lead to a reduction in the stainable iron stores of their liver [13]. In this work the *in vitro* iron mobilisation effects of DF at physiological pH in human and horse spleen ferritin and also in freshly prepared iron(III) precipitates were compared to those of L_1, L_2, L_3 maltol and mimosine, under the same conditions. Furthermore, repeated chelator incubations were used in order to identify mobilisation mechanisms of polynuclear and other iron forms which could be related to the iron chelation effects observed in iron loaded patients.

Experimental

Materials

L_1 and L_3 were prepared as previously described [4, 14]. The preparation of L_1 was based on a method of preparation of other pyridone derivatives [15]. L_2 was prepared from 2,3-dihydroxypyridine and methyl iodide [16]. Maltol was obtained from Aldrich, U.K.; DF from Ciba-Geigy, U.K.; Phosphate buffered saline (PBS) from Oxoid, U.K.; Mimosine and equine spleen ferritin from Sigma, U.K. (100 mg/ml, 16% (*w*/*v*) iron, 0.182 mM protein).

*Abbreviations: PBS, Phosphate buffered saline; L_1, 1,2-dimethyl-3-hydroxypyrid-4-one; L_2, 1-methyl-3-hydroxypyrid-2-one; DF, desferrioxamine; L_3, 1,4-dihydroxypyrid-2-one.

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Spectrophotometric Studies

In the spectrophotometric studies horse spleen ferritin (250 μl) was mixed with PBS (4.75 ml), and aliquots (0.75 ml) were placed in dialysis bags containing PBS (0.75 ml) and dialysed against the chelators (12 ml, 1×10^{-3} M, in PBS, pH 7.3) by continuous stirring at 37 °C in stoppered glass tubes. Ferric chloride (0.75 ml, 1×10^{-2} M) was added to PBS (0.75 ml), in dialysis bags and allowed to stand for 0.5 h, forming precipitates, which were dialysed against the chelators as above.

The amount of iron mobilised in the spectrophotometric studies were estimated from their extinction coefficients ϵ ($M^{-1} cm^{-1}$) as follows: Ferritin iron $\epsilon_{420} = 560$ [17] L_1–iron complex $\epsilon_{460} = 3600$; L_2–iron complex, $\epsilon_{510} = 3970$ [4], desferrioxamine–iron complex $\epsilon_{428} = 2770$ [7], maltol–iron complex $\epsilon_{410} = 3720$.

Iron Mobilisation Studies

Human spleen ferritin was isolated as previously described [18]. Its protein content was estimated at 1.67 mg/ml using the Lowry *et al.* method [19], and its iron content, which was determined by atomic absorption spectrophotometry following acid digestion with HNO_3 (23% *v/v*, 11 M), was found to be 3273 molecules of iron per ferritin molecule.

Iron release from horse spleen ferritin and iron(III) precipitates was estimated spectrophotometrically by taking the absorption spectra of the dialysates at different time intervals and from human spleen ferritin by measuring the amount of iron in the dialysates using atomic absorption spectrophotometry. Following the first 8 d of incubation, the dialysis bags were taken and dialysed overnight against PBS to remove the iron chelator complexes. The following day, the dialysis bags were reincubated with fresh chelator (12 ml, 1×10^{-3} M) and iron release was monitored as above for another 8 d. This process was repeated once more with new chelator solutions only in the case of horse spleen ferritin and the iron(III) precipitates.

Results

The release of iron from human and horse spleen ferritin, and iron(III) precipitate following repeated incubations with fresh chelator solutions is shown in Figs. 1–3 and Table I. The amount of iron released from ferritin differed between the chelators but in all the cases it was higher in the first 8 d incubation, less in the second and even less in the third. The chelators L_2, DF and maltol caused faster and quantitatively larger amounts of iron to be released from the iron(III) precipitate in comparison to horse spleen ferritin iron (Figs. 1 and 3) but for L_1 it was about the same for both iron forms. DF and L_1

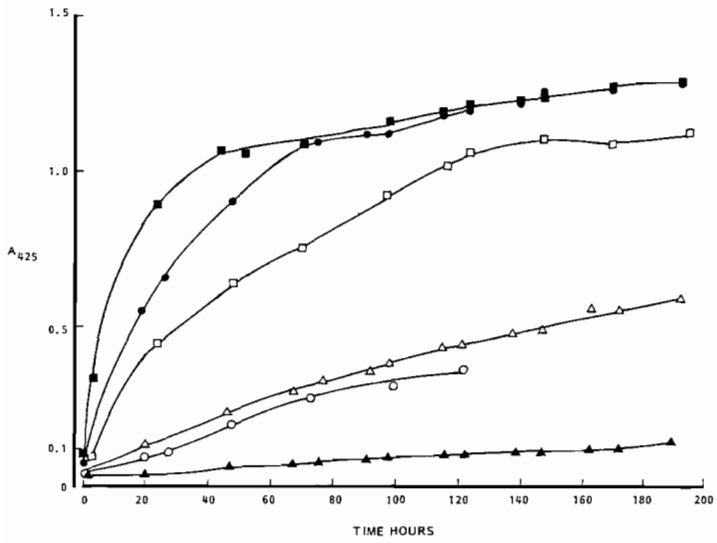

Fig. 1. Iron release from horse spleen ferritin and iron(III) precipitates by DF. Horse spleen ferritin (1.5 ml, 600 μg iron) (□) and two iron(III) precipitates (1.5 ml, 462 μg iron ■, ●), which were studied at two different periods, were all enclosed in dialysis tubing and continuously stirred at 37 °C against a DF solution (12 ml, 1×10^{-3} M) in stoppered glass tubes for 8 d. The progress of the reaction was monitored spectrophotometrically by measuring the change of absorbance at 425 nm. The amounts of iron released at the end of the 8 d incubations, estimated from the extinction coefficient of the DF iron complex ($\epsilon_{425} = 2770$) were 300 μg from ferritin (□) and 383 μg (■, ●) from the iron(III) precipitates. The dialysis tubings were then removed dialysed overnight against PBS and reincubated against a fresh DF solution as above. The amounts of iron released in the second 8 d period were 164 μg from ferritin (△) and the remaining 49 μg from the iron(III) precipitate (▲). The above procedure was repeated once more with ferritin only for further 5 d causing the release of the remaining 135 μg iron (○).

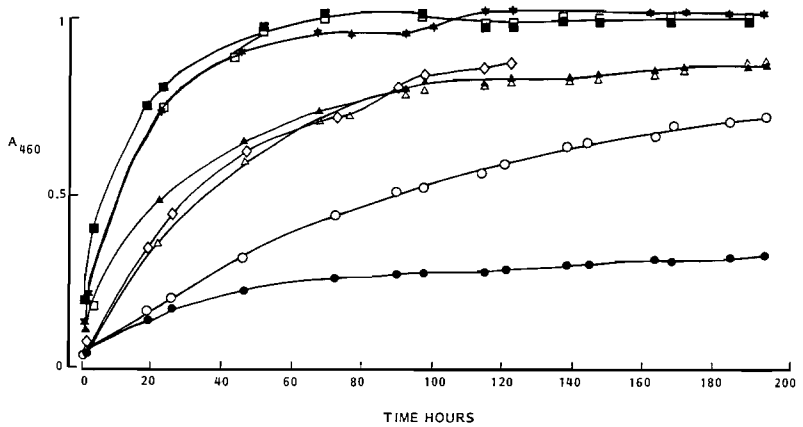

Fig. 2. Iron release from horse spleen ferritin and iron(III) precipitate by L_1. Two samples of horse spleen ferritin (1.5 ml, 600 μg iron) (□, ★) studied at two different periods and an iron(III) precipitate (1.5 ml, 462 μg iron) (■) were enclosed in dialysis tubing and treated with L_1 (12 ml, 1×10^{-3} M) using the same conditions and methods as for Fig. 1. The progress of the reactions was monitored in the dialysate at 460 nm. The amount of iron released in each incubation which was estimated using the extinction coefficient of its iron complex ($\epsilon_{460} = 3600$) in the first 8 d period, was found to be 210 μg in all three reactions. In the second 8 d period iron release was again the same (179 μg) in the iron(III) precipitate (▲) and one of the ferritins (△) and also approximately for the second ferritin incubation which was followed only for 5 d (◇). In the third 8 d period all the iron of the iron(III) precipitate (●) and almost all the iron (151 μg) from ferritin (○) was mobilised.

TABLE I. Iron Release from Human Spleen Ferritin Following Repeated Incubations with L_1, L_3, DF and Mimosine[a]

Chelator	Iron released (μg)	
	First 8 d period	Second 8 d period
L_1	84	58
L_3	80	58
DF	69	40
Mimosine	64	49

[a]Human spleen ferritin (1.67 mg/ml, 340 μg iron, 2 ml) enclosed in a dialysis tubing was stirred at 37 °C against a chelator solution (12 ml, 1×10^{-3} M) for 8 d, the dialysis tubing was then removed, dialysed against PBS and re-incubated with chelators of the same concentration for further 8 d. The amount of iron in the dialysates of both 8 d incubations for each chelator was estimated by atomic absorption spectrophotometry.

mobilised almost all the iron from horse spleen ferritin following the 3 × 8 d period incubations. All the iron(III) precipitate was also mobilised following a 3 × 8 d incubation period with L_1 but over the second 8 d incubation with DF (Figs. 1 and 2). L_2 was less effective than L_1 and DF, but more effective than maltol in iron mobilisation under the same conditions (Fig. 3). The release of iron from human spleen ferritin over the same incubation periods (2 × 8 d) caused by four chelators namely L_1, L_3, DF and mimosine and measured by atomic absorption spectrophotometry was also shown to decrease in the second 8 d period in comparison to the first 8 d period (Table I). L_1 and L_3 were more effective during these incubations than mimosine and DF. L_1 mobilised approximately the same amount of iron as L_3 and DF the same as mimosine (Table I).

Discussion

The release of iron from human and horse spleen ferritin and iron(III) precipitates following repeated incubations with chelators of the same concentrations was shown to decrease as the iron content of the polynuclear iron forms decreased. Although the amount of iron incubated with the chelators was well in excess of their binding capacity, the amount of iron released in the first or subsequent 8 d incubations was much smaller than the iron binding capacity of either the bidentate chelators which form 3 chelator:1 iron complex or of DF which forms 1:1 complex at physiological pH with mononuclear iron [4] (Figs. 1–3, Table I). This indicates that the depolymerisation rate of polynuclear iron cores decrease as the iron is mobilised and also that polynuclear iron complexes have affinity and are in competition with other ligands (or chelators) for mononuclear iron.

In this work chelators varied in their ability to mobilise polynuclear iron. DF mobilised the highest amount of iron from the iron(III) precipitate and the horse spleen ferritin in the first 8 d incubation, but this was just over 50% of its total iron binding capacity. Although the other chelators mobilised less iron, maltol and L_2 were as equally effective and L_1 more effective than DF in relation to the amount of iron chelated per binding capacity under the same

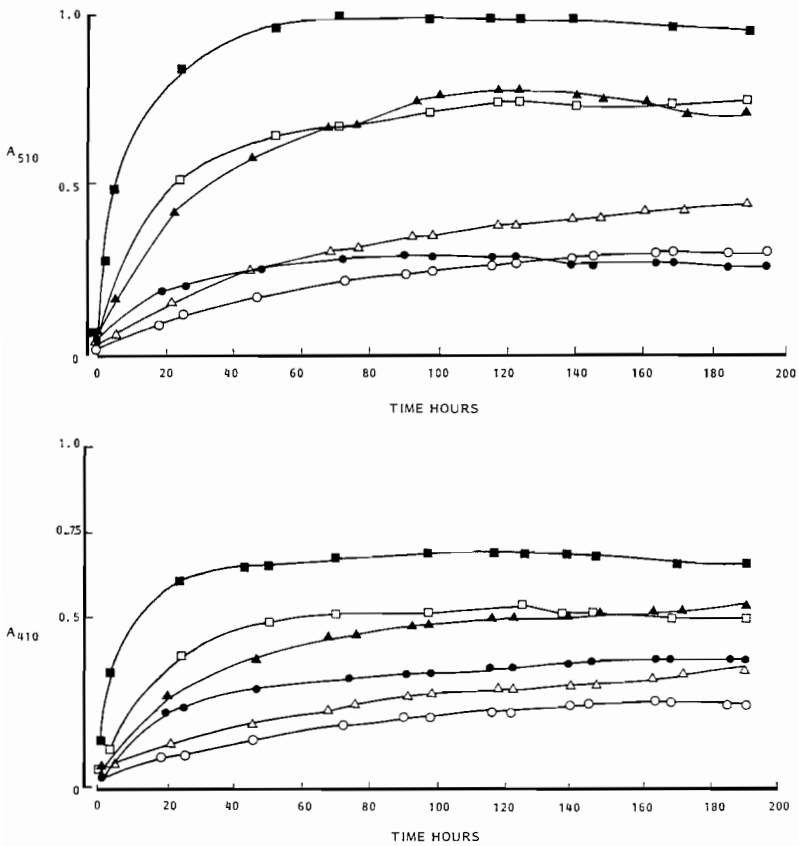

Fig. 3. Iron release from horse spleen ferritin by L_2 (top) and maltol (bottom). Two samples of horse spleen ferritin (1.5 ml, 600 μg iron) (□) and two iron(III) precipitates (1.5 ml, 462 μg iron) (■) were enclosed in dialysis tubing and each treated with either L_2 (top) or maltol (bottom) both at 1×10^{-3} M, 12 ml using similar methods and conditions as for Fig. 1. The amounts of iron released in the incubations were estimated from the extinction coefficients of their iron complexes (L_2, ϵ_{510} = 3970, maltol ϵ_{410} = 3720). Following three repeated incubations each of 8 d duration, L_2 caused the release of 143, 86 and 57 μg iron from ferritin and 181, 133 and 52 μg iron from iron(III) precipitate respectively and maltol 102, 72 and 51 μg iron from ferritin and 133, 113 and 78 μg iron from iron(III) precipitate over the same periods. – – –, △, ○, Represents the 2nd and 3rd 8 d ferritin; and – – –, ▲, ●, the 2nd and 3rd 8 d iron(III) precipitate incubation periods, respectively.

conditions. In the second 8 d incubation with horse spleen ferritin, L_1 mobilised more iron than DF. Similarly L_1 and L_3 were more effective and mimosine equally effective in comparison to DF in the mobilisation of iron from human spleen ferritin (Table I).

Although different kinetic and thermodynamic iron solubility constants govern the reactions of the iron forms with each chelator [14, 29], the faster solubilisation of the iron(III) precipitates in comparison to horse spleen ferritin, which was observed by all the chelators (Figs. 1–3), suggests that more soluble forms of iron were present in the former preparation [18]. This is supported by previous observations where it was shown that full iron precipitation from an iron solution attained a steady state in 200 h [20], and also from a ferritin iron reductive mobilisation study where iron mobilisation was faster with newly formed ferritin than a ferritin preparation of long term iron incubation [21]. It can be envisaged that similar forms of iron may occur *in vivo* and the amount of iron which could be mobilised by chelators at any instance would depend on the form and size of each of the following iron pools which have decreasing rate of solubility from left to right.

Mononuclear ⟶	Oligonuclear ⟶	Polynuclear
Low M_r Pool [22]		Ferritin, Haemosiderin

It should be noted that mononuclear iron in the form of haem is not available to the chelator but in the form of transferrin is partially available to some chelators and steady state in this later case is attained in a few hours [6].

Since most of the iron in iron overload diseases e.g. in β-thalassaemia is stored in a polynuclear form

[1] it can be suggested from these and other clinical results [23] that following the administration of a chelator e.g. DF or L_1, the amount of iron released would depend mainly on the polynuclear iron form and load of the patient [24–26] and that subsequent administrations would cause the excretion of less polynuclear iron because of the decrease in the solubility of the polynuclear iron pools remaining in the patient. Many factors however, could modify the iron binding properties of a chelator *in vivo* such as rapid excretion, bio-transformation, affinity for a different cellular iron pool [27], variation of iron turnover of different tissues, cells, proteins, etc. [14, 27–29].

In summary the following factors have been identified to effect the mobilisation of iron in this study: (a) form and (b) content of the iron source; (c) the iron solubilising ability; (d) the iron binding capacity and (e) the kinetic iron mobilisation efficacy of the chelator. The ability of the bidentate heteroaromatic chelators to mobilise increased amounts of iron from ferritin and the iron(III) precipitate, of levels equivalent or greater (e.g. L_1 and L_3) to those of DF increase the prospects for their or their analogs [11, 14, 28] use in the treatment of iron overload and other diseases of iron imbalance and toxicity.

Recently, the efficacy of L_1 in increasing the mobilisation and excretion of iron in iron loaded patients has been shown [26].

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