Biochem. J. (1986) 233, 299–302 (Printed in Great Britain)

Iron mobilization from ferritin using α-oxohydroxy heteroaromatic chelators

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Several α-oxohydroxy heteroaromatic chelators have been shown to mobilize iron from horse spleen ferritin. Although the reactions were slow, taking up to 3 days to reach completion, the amounts of iron mobilized were higher than those reported for other chelators. These results increase the prospects for the clinical use of α-oxohydroxy chelators in the treatment of iron overload.

INTRODUCTION

Many cellular processes are dependent on iron-containing proteins (Kontoghiorghes, 1982). The need for iron, coupled together with the toxic effects of 'free' iron in biological systems, causes organisms to keep this metal well confined and controlled. Ferritin is a water-soluble, hollow, spherical-shaped protein found in al mammalian cells and used for iron storage. Apo-ferritin has an M_r of 450000 and it can store up to 4500 atoms of iron(III) in a polynuclear oxyhydroxide phosphate complex (Gray, 1975). Iron transport in and out of the hollow sphere is though to be mediated through six hydrophobic and possibly eight other hydrophilic channels (Rice *et al.*, 1983). Iron uptake by ferritin *in vitro* has been shown to occur via an oxidation process of iron (II) (Crichton & Roman, 1977) or a low-M_r iron(III) complex deposition (Treffry & Harrison, 1979). Solubilization of the polynuclear ferritin iron core can be achieved either by reduction (Sirivech *et al.*, 1974; Dognin & Crichton, 1975; Crichton *et al.*, 1975; Munro & Linder, 1978) or by chelators (Pape *et al.*, 1968; Crichton *et al.*, 1980).

Iron mobilization from ferritin is of interest to those involved in the design and therapeutic use of iron chelators, e.g. the treatment of iron overload in regularly transfused thalassaemia patients, where excessive iron is accumulated in the liver, spleen, heart and other tissues in the form of ferritin and haemosiderin.

In this preliminary study, several α-oxohydroxy iron chelators have been studied for their ability to mobilize iron from ferritin at physiological pH. Some of these chelators were shown to mobilize iron from transferrin *in vitro* and from mice and rabbits *in vivo* (Kontoghiorghes, 1985).

EXPERIMENTAL

Materials

1,2-Dimethyl-3-hydroxypyrid-4-one (L_1) was prepared as previously described (Kontoghiorghes, 1982), by a method based on the preparation of other pyridone derivatives (Harris, 1976). For a different method of preparation of L_1, see also Yasue *et al.* (1970). 1-Methyl-3-hydroxypyrid-2-one (L_2) was prepared by the method of Mohrle & Weber (1970). Maltol was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., desferrioxamine mesylate (DFB) from Ciba–Geigy, Horsham, Sussex, U.K., phosphate-buffered saline (PBS) from Oxoid, Basingstoke, Hants., U.K., and equine spleen ferritin, from Sigma, Poole, Dorset, U.K. [100 mg/ml, 18% (w/v) iron] was used without further purification. The ferritin concentration (0.182 mM) was derived from the above information from Sigma.

Methods

Two different incubation methods in PBS, pH 7.3, were used to study iron mobilization from ferritin by chelators. In method A, the ferritin/chelator mixtures and in method B the diffusates of ferritin/chelator mixtures were examined spectrophometrically. In both cases, the iron content of ferritin and that of the chelator–iron complexes were estimated from their absorption coefficients [ϵ ($M^{-1} \cdot cm^{-1}$)] as follows.

Ferritin iron, $\epsilon_{420} = 560$ (Harrison *et al.*, 1974); L_1–iron complex, $\epsilon_{460} = 3600$; L_2–iron complex, $\epsilon_{510} = 3970$ (Kontoghiorghes, 1982); desferrioxamine–iron complex, $\epsilon_{428} = 2770$ (Crichton *et al.*, 1980); maltol–iron complex, $\epsilon_{410} = 3720$.

Ferritin/chelator mixture studies (method A)

Reactive mixtures (10 ml) were prepared in stoppered glass tubes by mixing ferritin with the chelators in a volume ratio of 3–5 μl of ferritin (0.182 mM) to 1 ml of chelator. Iron removal from ferritin was studied spectrophotometrically by measuring the change in absorbance of the ferritin/chelator mixture at different time intervals. The amount of the chelator–iron complex (CFe) formed was estimated at a selected wavelength of the visible absorbance spectra of the mixture in the vicinity of the $\lambda_{max.}$ of the iron–chelator complex by using the eqns. (1) and (2) (in the selected wavelength the

Abbreviations used: L_1, 1,2-dimethyl-3-hydroxypyrid-4-one; L_2, 1-methyl-3-hydroxypyrid-2-one; DFB, desferrioxamine mesylate; DHB, 2,3-dihydroxybenzoic acid; PAPHY, pyridine-2-aldehyde-2-pyridylhydrazone; NTA, nitrilotriacetic acid; 3,4-LICAMS, *NN'N''*-tris-(2,3-dihydroxy-5-sulphobenzoyl)-1,5,10-triazadecane; MECAM, *NN'N''*-tris-(2,3-hydroxybenzoyl)-1,3,5-triaminomethylbenzene.

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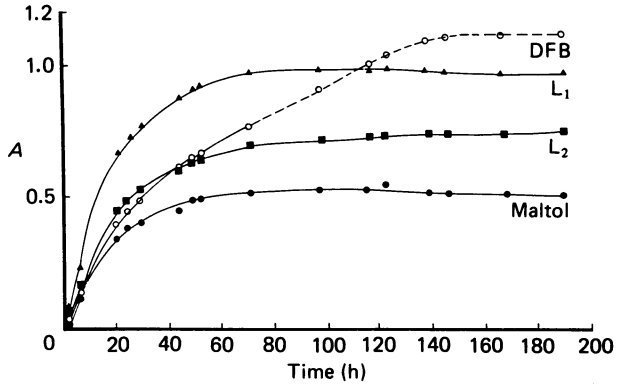

Fig. 1. Iron mobilization from ferritin using chelators

The reaction of the chelators (1 mM, 12 ml) with ferritin iron (0.59 mM) of ferritin/chelator mixture (1.5 ml) enclosed in a dialysis bag was studied by measuring the change in absorbance (A) of the chelator–iron complexes in the diffusate at 428 nm for desferrioxamine, at 460 nm for 1,2-dimethyl-3-hydroxypyrid-4-one, at 510 nm for 1-methyl-3-hydroxypyrid-2-one and at 410 for maltol. ---- indicates precipitation.

free non-bound chelator is colourless and its absorbance is close to zero):

$$A_m = A_{pf} + (A_{pi} - A_{pf}) \times \frac{\epsilon_{CFe}}{\epsilon_p} \quad (1)$$

$$A_{CFe} = A_m - A_{pf} \quad (2)$$

A_m is the absorbance of the mixture at time t, A_{pf} the absorbance of ferritin at time t, A_{pi} the absorbance of ferritin at zero time, A_{CFe} the absorbance of the chelator–iron complex, ϵ_{CFe} the absorption coefficient of the chelator–iron complex, ϵ_p the absorption coefficient of ferritin iron. All the parameters in eqns. (1) and (2) were measured at the same selected wavelength.

Ferritin dialysis studies (method B)

A chelator/ferritin solution enclosed in a dialysis bag (1.5 ml) was prepared and dialysed against a continuously stirred chelator solution (12 ml) in a stoppered glass tube. The volume ratio of ferritin to chelator was 2–4 μl of ferritin (0.182 mM) to 1 ml of chelator. The amount of iron removed from ferritin was estimated by measuring the absorbance of the iron–chelator complex formed in the diffusate at different time intervals.

RESULTS AND DISCUSSION

The α-oxohydroxy chelators have been shown to mobilize iron from ferritin at pH 7.3 in a slow reaction, taking up to 3 days to reach completion. During these reactions there was an increase in the absorbance of the mixtures at the wavelength regions that correspond to the $\lambda_{max.}$ of the chelator–iron complexes. The gradual formation of the iron complexes in all the incubations has indicated a common kinetic mobilization pattern characterized by an initial rapid removal of iron, followed by a slower mobilization rate and finally the end of the reaction as judged from the unchanged absorbance readings recorded over several days. All the α-oxohydroxy chelators formed clear, coloured iron complexes, in contrast with the desferrioxamine mixture, where precipitates were observed after 48 h of incubation (Fig. 1).

The amounts of iron mobilized by the chelators at 6 h, 24 h and after completion, varied significantly (Table 1) and were not related to $K_{sol.}$ (Schubert, 1964), the polynuclear iron solubility constant of the chelators [$\log(K_{sol.}\,L_1) = 9.6$; $\log(K_{sol.}\,L_2) = 10.1$; $\log(K_{sol.}$ maltol$) = 8.0$ (Kontoghiorghes, 1982), $\log(K_{sol.}$ DFB$) = 8.0$ (Schubert, 1964)]. The increased-temperature and the continuous-stirring procedure used in method B, but not in A, caused higher amounts of iron to be released from ferritin (Table 1).

Iron releases measured in the present study, with α-oxohydroxy chelators, were higher than those reported

Table 1. Iron mobilization from ferritin using α-oxohydroxy chelators

The reactions of the chelators with ferritin enclosed in a dialysis bag (method B) or in a solution mixture (method A) were carried out in PBS, pH 7.3. The concentration of iron in ferritin and of the chelator–iron complexes formed were estimated from their absorption coefficients.

Chelator	[Chelator] (mM)	[Iron] in ferritin (mM)	Iron mobilized (%) 6 h	24 h	> 72 h	Temp. (°C)	Method
1,2-Dimethyl-3-hydroxypyrid-4-one	1.00*	1.42	4.4	10.8		29	A
	3.80	1.74	13.9	31.4		37	B
	1.00	0.59	13.9	34.4	46.3	37	B
1-Methyl-3-hydroxypyrid-2-one	4.00	1.78	2.6	8.2		26	A
	3.80	1.74	11.1	22.2		37	B
	1.00	0.59	3.3	20.5	29.3	37	B
Maltol	4.00	1.78	1.2	3.7		26	A
	3.80	1.74	7.3	15.2		37	B
	1.00	0.59	5.0	18.1	23.9	37	B
Desferrioxamine	1.00	0.59	7.6	27.0		37	B

* In Tris/HCl/0.1 M-$NaClO_4$, pH 7.4.

Table 2. Iron mobilization from ferritin by known chelators reported previously

The percentages of iron mobilized from horse spleen ferritin were derived from the incubation of the chelators with (*a*) a mixture of ferritin ([Fe] = 2.54 mM) in Mops buffer (0.2 M, pH 7.4) at 37 °C in Crichton *et al.* (1980), (*b*) a mixture of ferritin ([Fe] = 0.5 mM) in Tris/HCl (0.05 M, pH 7.4) at 37 °C in Tufano *et al.* (1981), (*c*) ferritin ([Fe] = 125–36 μM) enclosed in a dialysis bag in Tris/HCl, (5 mM, pH 7.4) at 25 °C in Pape *et al.* (1968), and (*d*) ferritin, containing 1500 atoms of iron per molecule, in a dialysis bag in Tris/HCl (0.1 M, pH 7.4) at room temperature in Dognin & Crichton (1975).

Chelator	Concn. (mM)	Iron mobilized (%) 6 h	24 h	References
DHB	1.0		2.0	Crichton *et al.* (1980)
Rhodotorulic acid	1.0		11.5	Crichton *et al.* (1980)
PAPHY	1.0		4.0	Crichton *et al.* (1980)
DFB	1.0		10.0	Crichton *et al.* (1980)
DFB†	1.0	2.6		Tufano *et al.* (1981)
3,4-LICAMS	6.0	0.0		Tufano *et al.* (1981)
3,4-LICAMS†	6.0	5.0		Tufano *et al.* (1981)
MECAM‡	0.1	0.8		Tufano *et al.* (1981)
EDTA	10	0.6	1.0	Pape *et al.* (1968)
NTA	10	4.5	9.5	Pape *et al.* (1968)
Sodium citrate	10	0.3	0.4	Pape *et al.* (1968)
Glutathione	5.0	2.1*	7.0	Dognin & Crichton (1975)
Cysteine	5.0	4.7*	15.3	Dognin & Crichton (1975)
Ascorbate	5.0	3.3*	11.9	Dognin & Crichton (1975)
Ascorbate	100.0	14.0*	45.4	Dognin & Crichton (1975)

* Percentage of iron removed in 5 h.
† In the presence of 6.0 mM-ascorbate.
‡ In dimethyl sulphoxide and water.

previously for other known chelators over the same periods of incubation with ferritin, even when ascorbic acid was used in the latter case to facilitate iron removal (Table 2). Although iron mobilization from ferritin by chelators is not clearly understood, there are several observations that may help to explain the mode of their action. For example, the catechol-type chelators 3,4-LICAM and MECAM, which were found previously to mobilize iron from transferrin (Carrano & Raymond, 1979), failed to mobilize iron from ferritin, except when ascorbic acid was present (Tufano *et al.*, 1981). Steric hindrances due to the large size of the chelators or their iron complexes in comparison with the size of the channels may be the cause of their ineffectiveness. It seems that iron mobilization from ferritin by chelators depends on several factors such as: (*a*) the ability of chelators to solubilize polynuclear iron (Pape *et al.*, 1968); (*b*) the presence of mediators such as ascorbic acid (Table 2); and (*c*) the size, charge and other structural features such as lipid/water solubility (Kontoghiorghes, 1982), of the chelator and its iron complex.

It was also found that the rates of iron chelation at physiological pH of different forms of iron(III) vary considerably, as suggested by Schubert (1964). When the α-oxohydroxy chelators were mixed with aqueous $FeCl_2$ or $FeCl_3$ solutions, ferric complexes were formed almost instantaneously, but the chelation of iron from transferrin and ferritin took up to 3 h (Kontoghiorghes, 1982) and 3 days respectively to reach completion. Iron-solubilization rates similar to those seen with ferritin iron were also observed when the α-oxohydroxy chelators were incubated with iron(III) precipitate (G. J. Kontoghiorghes, unpublished work). In the present study the polynuclear-complex form of the ferritin iron core and not the size of the ferritin channels seems to be the cause of the slow reactions of the α-oxohydroxy chelators with ferritin.

It is suggested, on the basis of these results, that ferritin iron is not easily accessible to chelators, and this may reflect the difficulties associated with the prolonged treatment of iron overload in thalassaemia. In this respect, a continuous chelation will be more advantageous in mobilizing ferritin and other polynuclear forms of iron from thalassaemia patients than the present 8–10 h/day desferrioxamine treatment. The ferritin-dialysis method (method B) was found to be a useful procedure for screening chelators and the ability of the α-oxohydroxy chelators to mobilize substantial amounts of iron from ferritin increases the prospects of the use of some of these, or their analogues, in the treatment of iron overload and other diseases of iron imbalance.

I thank the U.K. Thalassaemia Society and the British Technology Group for financial assistance, Professor A. V. Hoffbrand, Professor E. R. Huehns, Dr. R. C. Hider and Dr. M. T. Wilson for helpful discussions, and Miss Josephine Martin for typing the manuscript.

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Received 16 August 1985/30 October 1985; accepted 1 November 1985