FERRITIN IRON MOBILISATION BY CHELATING AGENTS

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Received 17 December 1979

1. Introduction

In a large number of eukaryotes iron is stored in a soluble non-toxic form in ferritin. The iron is deposited in the interior of an approximately spherical protein shell, predominantly as ferric oxyhydroxide (reviewed [1,2]). A number of mechanisms have been proposed for ferritin iron release involving low molecular weight chelators [3,4], enzyme systems such as xanthine oxidase [5] or a ferriductase [6], and more recently by reduction of the ferric iron by reduced flavins [7–10], followed by chelation by a suitable complexing agent.

The direct mobilisation of ferritin iron by chelators does not seem to be a likely mechanism in vivo. The development of a chelator that could directly mobilise storage iron in a form that could be easily eliminated from the body, would be of considerable interest in the treatment of disorders of iron metabolism characterized by secondary iron overload. Such a clinical situation is encountered in Cooley's anaemia, where the consequence of prolonged blood transfusion is the accumulation of finally toxic levels of iron in liver, heart and other tissues.

We have examined the release of ferritin iron in vitro by the iron chelators: 2,2′-bipyridyl, desferrioxamine B, rhodotorulic acid, 2,3-dihydroxybenzoate, and the bipyridyl analogue, pyridine-2-aldehyde-2-pyridyl hydrazone (Paphy). The effects of a number of molecules that might assist in mediating iron release between the ferritin iron core and the chelators was also studied.

2. Materials and methods

Horse spleen ferritin was obtained from Boehringer (Mannheim), Mops (morpholine propane sulphonic acid) from Serva (Heidelberg), 2,3-dihydroxybenzoate and Paphy (pyridine-2-aldehyde-2-pyridyl hydrazone) from Aldrich (Beerse). Desferrioxamine B was a gift from Ciba-Geigy (Basle) and rhodotorulic acid was a gift from Dr A. Cerami, Rockefeller University, New York). FMN was from Serva (Heidelberg) and NADH from Boehringer (Mannheim). Ascorbate and EDTA were from Merck (Darmstadt).

Ferritin protein and iron concentration were determined by amino acid analysis and by the method of [11], respectively. The λ_{max} for the various iron chelators was determined in 200 mM Mops buffer (pH 7.4) as in [11]. Iron release from ferritin was followed at the appropriate λ_{max} by incubation of ferritin (final conc. 10^{-6} M in protein) in 200 mM Mops buffer (pH 7.4) together with the chelator at 1 mM final conc. in a waterbath at 37°C. The samples, in 1 ml final vol. in disposable cuvettes (Kartell, Milan) were removed at appropriate time intervals and the amount of iron released determined spectrophotometrically.

The mean ± SE for 5 samples was determined. In the experiments with FMN, ascorbate and EDTA, these latter substances were introduced into the reaction medium at 1 mM final conc. The appropriate controls which were used for FMN with bipyridyl and Paphy are described in [11].

3. Results and discussion

Iron release from ferritin by the five chelators studied are presented in fig.1. We can readily establish that rhodotorulic acid and desferrioxamine B (which are both trihydroxamic acids) release the greatest amounts of iron, and over 24 h incubation at 37°C, liberate 250 g atoms and 225 g atoms iron/ferritin molecule, respectively. Of the other chelators only

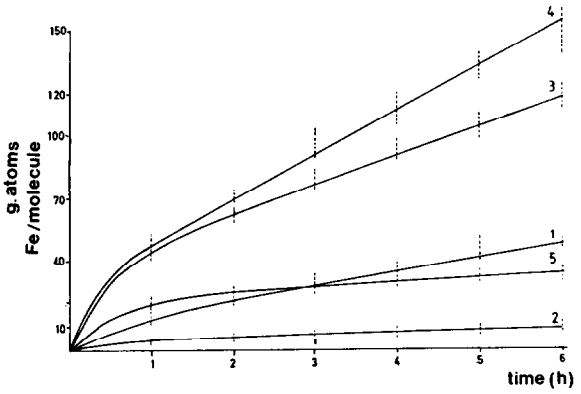

Fig.1. Iron release from ferritin by chelators. Iron release from horse spleen ferritin was measured as in section 2 and is expressed as g atoms Fe/molecule released at the appropriate time interval; the standard errors are indicated by error bars. The chelators employed (at 1 mM final con.) are: (1) Paphy; (2) bipyridyl; (3) desferrioxamine B; (4) rhodotorulic acid; (5) 2,3-dihydroxybenzoate.

Paphy gives a reasonable release of 85 g atoms/ferritin molecule in 24 h whereas the release of iron by 2,3-dihydroxybenzoate does not evolve significantly from the plateau value observed in fig.1 even after 24 h incubation.

When we turn to the effects of the mediators, we can consider first the effects of the reducing agent ascorbate and the complexant EDTA; the results are presented in table 1. A slight positive effect was observed with desferrioxamine B in the presence of ascorbate, and a more important increase corresponding to 30% after 6 h with EDTA. With rhodotorulic acid, ascorbate also had a positive effect on iron mobilisation, but EDTA inhibited iron release markedly; after 6 h the amount of iron released in the presence of EDTA was reduced to 40% of the control value. The stimulatory effect of ascorbate could be explained by reduction of ferritin iron and its release from ferritin as a Fe II–ascorbate complex which thereafter is transfered to the hydroxamic acid. EDTA might act by chelating the ferritin iron directly: the transfer of iron from the Fe II–EDTA complex to the hydroxamic acid would be determined by the relative stability constants of the Fe-EDTA and Fe-hydroxamate and by the kinetics of iron exchange between the two chelators.

For bipyridyl and Paphy the effects of ascorbate and EDTA were very similar. For both chelators, ascorbate considerably increased ferritin iron release; after 6 h a 7-fold increase was observed for bipyridyl and a 2-fold increase for Paphy. This presumably reflects the preferential binding by these chelators of Fe II. EDTA virtually eliminated iron mobilisation by Paphy and bipyridyl, consistent with the higher affinity of EDTA for Fe III compared to the pyridinium derivatives.

Both ascorbate and EDTA inhibited iron release from ferritin by 2,3-dihydroxybenzoate, although the effect of EDTA was much greater than that of ascorbate.

For FMN at 1 mM final conc. the results are presented in table 2. It should be emphasized that all of the results were obtained with continuous illumination of the samples by standard strip lighting during the period of incubation. The results can be divided into 3 groups:

Table 1
Effect of ascorbate and EDTA on iron release from ferritin by chelating agents

Chelator	Iron release (g atom/molecule of ferritin) after incubation					
	No addition		+ Ascorbate		+ EDTA	
	2 h	6 h	2 h	6 h	2 h	6 h
Desferrioxamine B	62.1	116.8	71.9	136.1	83.1	154.5
Rhodotorulic acid	73.9	147.7	87.5	179.3	59.3	63.2
2,3-Dihydroxybenzoate	24.4	35.5	18.9	28.0	12.7	15.5
Bipyridyl	5.7	9.7	35.2	71.4	2.4	2.8
Paphy	22.4	49.3	52.0	131.5	1.5	6.1

In all experiments, the final concentration of ascorbate, of EDTA and of the chelator was 1 mM in 200 mM Mops buffer (pH 7.4). All results were obtained at 37°C with continuous illumination

Table 2
Effect of FMN on iron release from ferritin by chelating agents

Chelator	Iron release (g atom/molecule ferritin) after incubation		
	2 h	6 h	24 h
Desferrioxamine B	11.7	12.3	12.6
Rhodotorulic acid	21.9	21.5	22.3
2,3-Dihydroxybenzoate	26.1	26.0	26.8
Bipyridyl	15.8	41.3	116.5
Paphy	48.5	89.3	122.0

In all experiments the final concentration, both of FMN and of the chelator, was 1 mM in 200 mM Mops buffer (pH 7.4). All results were obtained at 37°C with continuous illumination

(1) Desferrioxamine B and rhodotorulic acid which, although the most effective chelators of ferritin iron in the absence of mediators, are almost completely inhibited by 1 mM FMN. The small amount of ferritin iron released in the course of the 1 h incubation does not evolve over the next 24 h. Therefore, either the FMN (probably in a reduced form) blocks access of the chelators to the sites of iron mobilisation, or else that the presence of small amounts of reduced FMN generates Fe II on the sites of mobilisation, and that the chelators (which certainly prefer Fe III to Fe II) do not have a sufficient affinity for the Fe II to be able to displace it from the sites.

(2) Bipyridyl and Paphy: Despite the problems associated with compensating for the absorption of photoreduced FMN in the assay system (see [11]) we observed a substantial enhancement of iron mobilisation by these 2 chelators in the presence of FMN, and of light; no difference, compared to control samples without flavin, was observed in the absence of light (R. R. C., F. R. unpublished). Since both bipyridyl and Paphy are good Fe II chelators (and since in the case of bipyridyl, the Fe III complex is not detected at the wavelength used), the increased iron release observed in the presence of FMN must reflect photoreduction of some of the FMN accompanied by reduction of ferritin iron. This is in complete accord with the observations [7–10] that ferritin iron is most effectively mobilised by reduction with reduced flavins prior to its complexation.

(3) For 2,3-dihydroxybenzoate a slight inhibition was observed. It should however be emphasized that, whereas this chelator rapidly releases some 40 g atoms iron/ferritin molecule (fig.1), no further iron release is observed.

In conclusion we have found that of the iron chelators currently employed for the treatment of iron overload (desferrioxamine B, rhodotorulic acid and 2,3-dihydroxybenzoate) the two hydroxamic acid derivatives are effective in ferritin iron mobilisation, but are almost completely inhibited in the presence of 1 mM FMN. Since there are good reasons to believe that the release of iron in vivo involves preliminary reduction of the ferritin iron by a reduced flavin, and that ferritin prepared by standard procedures [1,12] contains a flavin (J. C. M., R. R. C., unpublished), the mobilisation of ferritin iron by desferrixoamine B and by rhodotorulic acid in vivo may be inhibited, and that iron release by these chelators in vivo most probably reflects the capture of iron from a transit pool situated between intracellular storage iron (ferritin) and extracellular transport iron (transferrin) as suggested in [13,14]. Dihydroxybenzoate does not seem to be a likely candidate for direct mobilisation of ferritin iron in vitro. In contrast, chelators of Fe II such as bipyridyl and Paphy are well suited to take advantage of the reduction of ferritin iron by reduced flavins and may represent a potential approach to the direct mobilisation of ferritin iron in vivo. In cultured rat fibroblasts, bipyridyl, desferrioxamine B and 2,3-dihydroxybenzoate substantially diminish the intracellular accumulation of transferrin iron [15]. The study, in parallel, of in vitro and in vivo ferritin iron mobilisation may offer the most rational and appropriate method for the development of new iron chelators.

Acknowledgements

We thank Professor Tony Cerami for the gift of rhodotorulic acid, Ciba-Geigy (Basle) for the gift of desferrioxamine B, Karin Schanck for amino acid analyses and Institut pour l'Ecounragement de la Recherche Scientifique dans l'Industrie et l'Agriculture for a Bourse de Spécialisation to F. R.

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