Biochem. J. (1986) 234, 727–731 (Printed in Great Britain)

Formation of hydroxyl radicals in the presence of ferritin and haemosiderin

Is haemosiderin formation a biological protective mechanism?

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Horse spleen and human spleen ferritins increase the formation of hydroxyl radicals ('OH) at both pH 4.5 and pH 7.4 in reaction mixtures containing ascorbic acid and H_2O_2. The generation of 'OH is inhibited by the chelator desferrioxamine. Human spleen haemosiderin also accelerates 'OH generation in identical reaction mixtures, but is far less effective (on a unit iron basis) than ferritin under all reaction conditions. It is proposed that conversion of ferritin into haemosiderin in iron overload is biologically advantageous in that it decreases the ability of iron to promote oxygen-radical reactions.

INTRODUCTION

It is well established that iron overload causes substantial tissue damage and predisposes to the development of hepatoma (McLaren *et al.*, 1983). Iron is known to accelerate lipid peroxidation, and also formation of the highly reactive hydroxyl radical ('OH) from H_2O_2 in the presence of a reducing agent, such as superoxide ($O_2^{\cdot-}$) (McCord & Day, 1978; Halliwell, 1978). Available iron complexes, measurable by the bleomycin assay, are effective promoters of radical reactions (Floyd, 1983; Flitter *et al.*, 1983) and are often present in the plasma of iron-overloaded patients (Gutteridge *et al.*, 1985). Ferritin is also effective in promoting lipid peroxidation (Gutteridge *et al.*, 1983; O'Connell *et al.*, 1985) and $O_2^{\cdot-}$-dependent 'OH radical formation (Bannister *et al.*, 1984; Carlin & Djursater, 1984), probably because $O_2^{\cdot-}$ mobilizes iron from ferritin and this iron can then participate in the iron-catalysed Haber–Weiss reaction (Biemond *et al.*, 1984; Thomas *et al.*, 1985). Ascorbate stimulates lipid peroxidation in the presence of ferritin at pH 7.4, suggesting that ascorbate can also mobilize redox-active iron from this protein (Gutteridge *et al.*, 1983; O'Connell *et al.*, 1985).

In normal liver ferritin is the major iron protein present, but in iron overload haemosiderin predominates (Selden *et al.*, 1980*a*). Haemosiderin accumulates in lysosomes, especially in the liver, and a close positive correlation has been shown between enhanced lysosomal fragility and liver haemosiderin content (Selden *et al.*, 1980*a*). O'Connell *et al.* (1985) showed that iron was released from haemosiderin at pH 4.5 and could promote lipid peroxidation, whereas ascorbate was required to observe haemosiderin-dependent lipid peroxidation at pH 7.4. Hence excessive haemosiderin accumulation within tissues could provoke lysosomal damage by increasing lipid peroxidation (Selden *et al.*, 1980*b*). However, on a unit iron basis, haemosiderin was in fact less effective than ferritin in promoting lipid peroxidation (O'Connell *et al.*, 1985).

'OH can also damage lysosomal membranes (Fong *et al.*, 1973) and most other biomolecules (Halliwell & Gutteridge, 1984). Therefore in the present paper we have compared the ability of ferritin and haemosiderin to promote 'OH generation under physiological conditions. The 'OH radicals were detected by their ability to degrade deoxyribose (Gutteridge, 1981; Halliwell & Gutteridge, 1981) and to hydroxylate aromatic compounds with the formation of specific hydroxylated products (Moorhouse *et al.*, 1985).

MATERIALS AND METHODS

Horse spleen ferritin (0.16 mg of Fe/mg of protein) was from Calbiochem and xanthine oxidase from Sigma Chemical Co. Human haemosiderin (Weir *et al.*, 1984) and ferritin (0.29 mg of Fe/mg of protein) (Huebers *et al.*, 1974) were isolated from the spleens of iron-overloaded human thalassaemic patients. Haemosiderin was freeze-dried and stored desiccated at room temperature, solutions (about 10 mM in iron) being prepared in 20 mM-tetramethylammonium hydroxide (Weir *et al.*, 1984) as required. Haemosiderins B (2.17 mg of Fe/mg of protein) and G (7.27 mg of Fe/mg of protein) are preparations from two different human spleens. Desferrioxamine (as Desferal) was obtained from CIBA–Geigy. Iron content of proteins was determined by atomic absorption (Selden & Peters, 1979).

Deoxyribose degradation was followed essentially as described by Halliwell & Gutteridge (1981); detailed reaction conditions are given in Table legends. Aromatic hydroxylation with phenol as substrate was performed as described by Grootveld & Halliwell (1986), with salicylate as substrate as described by Moorhouse *et al.* (1985). Bovine erythrocyte superoxide dismutase was obtained from Sigma Chemical Co.; units of enzyme activity are as defined in the cytochrome *c* assay (McCord & Fridovich, 1969).

RESULTS

Bannister *et al.* (1984) reported that incubation of human ferritin with an $O_2^{\cdot-}$-generating system produced $^{\cdot}OH$ radicals, identified by spin trapping. In confirmation of these results, we found that incubation of horse spleen ferritin at pH 7.4 with an $O_2^{\cdot-}$-generating system [hypoxanthine plus xanthine oxidase, described in Richmond *et al.* (1981)] produced a reactive species that converted salicylate into 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and catechol (results not shown). These products are exactly as expected from attack of $^{\cdot}OH$ radical on the salicylate molecule (Moorhouse *et al.*, 1985).

Ascorbate can sometimes replace $O_2^{\cdot-}$ as an iron-reducing reagent in systems generating $^{\cdot}OH$ radicals (Winterbourn, 1979). Table 1 shows that incubation of horse spleen ferritin with ascorbate produced a reactive species that could hydroxylate salicylate to form the above mixture of products at both acid (pH 4.5) and alkaline (pH 7.4) pH values, although the ferritin was not as reactive as an equimolar amount of iron in the form of $FeCl_3$. An equal amount of haemosiderin iron was less active in forming hydroxylated products. The haemosiderin was dissolved in 20 mM-tetramethylammonium hydroxide, but addition of this reagent to the ferritin and $FeCl_3$ assays at the final concentration that would have been present in the haemosiderin assay did not change the results obtained. Omission of ascorbate from the reaction mixtures completely abolished the hydroxylation seen at pH 7.4 with ferritin and haemosiderin, and decreased that seen at pH 4.5 by 80–90% (results not shown).

That the hydroxylating species formed is indeed $^{\cdot}OH$ is suggested, not only by the hydroxylation pattern observed, but also by the effect of $^{\cdot}OH$ scavengers such as mannitol, formate and Tris. Table 2 shows that these inhibited the formation of hydroxylated products. Indeed, when experiments were carried out in Tris buffer, pH 7.4, the observed hydroxylations were diminished considerably (Table 1). These experiments, and those in unbuffered reaction mixtures, were carried out in order to show that phosphate buffer was not essential for $^{\cdot}OH$ production.

$^{\cdot}OH$ radicals can also be measured by their ability to degrade the sugar deoxyribose into a product that reacts with thiobarbituric acid to form a chromogen (Gutteridge, 1981). Table 3 shows a typical set of results obtained when ferritin and haemosiderin were included in reaction mixtures containing H_2O_2 and ascorbate. It may be seen that ferritin and haemosiderin accelerated deoxyribose degradation, although ferritin was less effective than an equimolar amount of $FeCl_3$, and haemosiderin was less effective than ferritin. The amount of deoxyribose degradation observed with haemosiderin or ferritin increased with iron concentration in the range 0–10 μM, but then reached a maximum. Control experiments again showed that the tetramethylammonium hydroxide in which the haemosiderin preparations were dissolved did not affect the results, in that this reagent, at the final concentration present in the haemosiderin experiments, did not affect deoxyribose degradation promoted by ferritin or $FeCl_3$. Omission of ascorbate at pH 7.4 abolished deoxyribose degradation induced by the proteins, and severely diminished the degradation seen at pH 4.5 (Table 3).

Human spleen ferritin was also effective in promoting deoxyribose degradation (Table 4). Deoxyribose degradation by ferritin or haemosiderin was decreased by the $^{\cdot}OH$ scavengers mannitol, formate and ethanol and by the iron-chelating agent desferrioxamine, but not by superoxide dismutase.

DISCUSSION

In this paper we have shown that a mixture of ascorbate, H_2O_2 and ferritin produces $^{\cdot}OH$ radicals at pH 7.4. Omission of ascorbate from the reaction mixture abolishes $^{\cdot}OH$ production. Presumably one function of the ascorbate is to mobilize iron from the ferritin; this iron then participates in a redox cycle with ascorbate and H_2O_2 to generate $^{\cdot}OH$. Hence $^{\cdot}OH$ generation is decreased by desferrioxamine, which chelates iron in a

Table 1. Aromatic hydroxylation by ferritin and haemosiderin

Reaction mixtures contained, in a total volume of 1.2 ml, the following reagents at the final concentration stated: salicylate (1 mM), H_2O_2 (1.4 mM), ascorbate (100 μM), iron (10 μM) and, where indicated, a buffer system (10 mM-KH_2PO_4/KOH, pH 7.4; 20 mM-acetic acid/sodium acetate, pH 4.5; 5 mM-Tris/HCl, pH 7.4). The pH of unbuffered reaction mixtures was approx. 3.6. Reaction mixtures were incubated at 37 °C for 30 min and the amounts of hydroxylated products formed (2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and catechol) were determined electrochemically after h.p.l.c. separation (Moorhouse *et al.*, 1985). Results are expressed as final concentrations of all hydroxylated products in the reaction mixture after correction for control incubations to which no iron had been added. Haemosiderins B and G are preparations obtained from two different human spleens. Results varied by 10% or less in three different experiments.

Form of iron added to reaction mixture (10 μM)	Total amount of hydroxylated products formed (μmol/litre)			
	Unbuffered system	Acetate, pH 4.5	Phosphate, pH 7.4	Tris, pH 7.4
$FeCl_3$	27	13	6	4
Haemosiderin B	2	2	1	< 1
Haemosiderin G	6	3	1	< 1
Horse spleen ferritin	19	5	5	4

Table 2. Aromatic hydroxylation by ferritin and haemosiderin

Reaction mixtures were as described in the legend to Table 1 except that incubation was for 2 h. The studies below were carried out in acetate buffer, pH 4.5, but similar results were obtained in systems containing other buffers. The ·OH scavengers were added to the reaction mixtures at the final concentrations stated.

Form of iron added to reaction mixture (10 μM)	Other reagent added	Inhibition of hydroxylated product formation (%)
$FeCl_3$	None	0 (corresponds to 43 μM hydroxylated product)
	10 mM-Mannitol	85
	50 mM-Mannitol	96
	10 mM-Formate	88
	50 mM-Formate	96
	10 mM-Tris	23
Horse spleen ferritin	None	0 (corresponds to 35 μM)
	10 mM-Mannitol	72
	50 mM-Mannitol	95
	10 mM-Formate	82
	50 mM-Formate	98
	10 mM-Tris	18
Haemosiderin G	None	0 (corresponds to 12 μM)
	10 mM-Mannitol	63
	50 mM-Mannitol	87
	10 mM-Formate	70
	50 mM-Formate	90
Haemosiderin B	None	0 (corresponds to 13 μM)
	10 mM-Mannitol	62
	50 mM-Mannitol	88
	10 mM-Formate	72
	50 mM-Formate	90
	10 mM-Tris	12

Table 3. Deoxyribose degradation by ferritin and haemosiderin

Reaction mixtures contained, in a total volume of 1.2 ml, the following reagents at the final concentrations stated: ascorbate (100 μM), H_2O_2 (1.44 mM), deoxyribose (2.8 mM), iron (10 μM) and, where indicated, a buffer system (10 mM-KH_2PO_4/KOH pH 7.4; 20 mM-acetic acid/sodium acetate, pH 4.5). The pH of unbuffered reaction mixtures was approx. 3.6. Tubes were incubated at 37 °C for 60 min and the deoxyribose degradation was measured as formation of thiobarbituric acid-reactive material at 532 nm as described by Halliwell & Gutteridge (1981). Results are given after correction for the control rate of deoxyribose degradation in each system, which was due to iron contamination of the reagents (it could be suppressed by the chelating agent desferrioxamine). Actual absorbance values varied by 15% or less in nine separate experiments, and in all experiments the order of effectiveness was $FeCl_3$ > ferritin > haemosiderin. A typical experimental result is shown.

Form of iron added to reaction mixture	Extent of deoxyribose degradation (A_{532}/h)		
	Unbuffered system	Acetate, pH 4.5	Phosphate, pH 7.4
$FeCl_3$	0.700	0.392	0.404
$FeCl_3$ (ascorbate omitted)	0.146	0.186	0.175
Horse spleen ferritin	0.455	0.384	0.371
Horse spleen ferritin (ascorbate omitted)	0.045	0.065	0.002
Haemosiderin B	0.111	0.301	0.016
Haemosiderin B (ascorbate omitted)	0.003	0.052	0.000
Haemosiderin G	0.189	0.271	0.021
Haemosiderin G (ascorbate omitted)	0.008	0.066	0.000

form that does not normally participate in radical reactions (Gutteridge *et al.*, 1979; Halliwell, 1985). Both human and horse spleen ferritins generate ·OH under our reaction conditions. Haemosiderin also promotes ·OH generation at pH 7.4, but to a much smaller extent than ferritin. For the most direct comparison, it should be noted that one of the haemosiderin preparations (G) had come from the same spleen as the human ferritin used.

At pH 4.5, an approximate value for intralysosomal pH, ferritin promoted a limited ·OH generation in the

Table 4. Deoxyribose degradation by ferritin and haemosiderin

Reaction mixtures, buffered at pH 7.4 with phosphate or at pH 4.5 with acetate, were as described in the legend to Table 3. Other reagents were added to give the final concentrations stated. —, Not tested. Actual absorbance values varied by 30% or less in four separate experiments, but the calculated percentage inhibitions varied by no more than 5% and haemosiderin was always less effective than ferritin. A typical experimental result is shown.

Form of iron added to reaction mixture (10 μM final iron concentration)	Other reagent added	Extent of deoxyribose degradation (A_{532}/h)	
		pH 4.5	pH 7.4
Human spleen ferritin	None	0.439	0.224
	Desferrioxamine (20 μM)	0.112	0.048
	Mannitol (1 mM)	0.382	0.136
	Mannitol (10 mM)	0.040	0.045
	Sodium formate (1 mM)	0.352	0.124
	Sodium formate (10 mM)	0.022	0.025
	Ethanol (10 mM)	0.074	0.045
	Superoxide dismutase (233 units/ml)	0.444	0.267
Horse spleen ferritin	None	0.490	0.312
	Desferrioxamine (20 μM)	0.268	0.000
	Mannitol (1 mM)	0.383	0.253
	Mannitol (10 mM)	0.074	0.094
	Sodium formate (1 mM)	0.338	0.218
	Sodium formate (10 mM)	0.071	0.086
	Ethanol (10 mM)	0.082	0.118
	Superoxide dismutase (330 units/ml)	0.495	0.345
Human spleen haemosiderin (haemosiderin G)	None	0.138	—
	Desferrioxamine (200 μM)	0.018	—
	Desferrioxamine (20 μM)	0.029	—
	Mannitol (1 mM)	0.045	—
	Mannitol (10 mM)	0.025	—

absence of ascorbate. O'Connell *et al.* (1985) have shown that Fe(III) is released from ferritin at this pH, and Fe(III) can react with H_2O_2 to form $^{\cdot}OH$ (Gutteridge, 1985; Table 3). Added ascorbate will increase the rate of iron mobilization from the protein and will also facilitate $^{\cdot}OH$ formation by reducing Fe(III) to Fe(II). Again, ferritin was less effective than $FeCl_3$ on a unit iron basis, and haemosiderin was less effective than ferritin.

Iron in the form of haemosiderin is far less active in promoting lipid peroxidation (O'Connell *et al.*, 1985) or $^{\cdot}OH$ formation (present paper) than is ferritin-bound iron either at pH 7.4 or at acidic pH values, such as exist within lysosomes. It could thus be argued that conversion of ferritin into haemosiderin is biologically advantageous, in that it would diminish the occurrence of oxygen-radical reactions during iron overload. Nevertheless, the iron in haemosiderin can itself participate in radical reactions, and so an excessive accumulation of this protein within lysosomes would be expected to damage their membranes. It may also be that radical reactions promoted by ferritin iron are actually involved in the formation of haemosiderin from ferritin.

We are grateful to the Arthritis and Rheumatism Council, Medical Research Council, Sports Council and the Wellcome Trust for financial support. B.H. is a Lister Institute Research Fellow.

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Received 19 November 1985/20 December 1985; accepted 31 December 1985