Iron release from ferritin and its sensitivity to superoxide ions differs among vertebrates

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The influence of the superoxide-generating system, xanthine oxidase, on the release of iron from various vertebrate ferritins was determined both in the presence and absence of superoxide dismutase. The initial rate of iron release in the presence of this system was higher for ferritins from human, trout and rat liver than for those from lamprey liver and horse spleen. The proportion of this iron release that was superoxide-dependent in the case of rat, human and trout ferritins was 92, 86 and 84 % respectively, whereas no such superoxide-dependent iron release occurred from the ferritins of lamprey liver and horse spleen. On the other hand, the rate of superoxide-independent iron release was of comparable magnitude for all of the species examined. The rate of superoxide-dependent iron release was related neither to the iron:protein ratios nor to the subunit size of the ferritins. However, it is significant that the ferritins with a high rate of superoxide-dependent iron release came from tissues known to be susceptible to iron damage. It is thus proposed that the resistance of lamprey liver ferritin to the mobilization of iron by superoxide ions accounts in part for the tolerance of the lamprey liver to high iron loads.

INTRODUCTION

Iron is an essential nutrient for all vertebrates [1]. In healthy tissues, it occurs predominantly in haemoglobin, myoglobin, catalytically active proteins and in the iron-storage proteins ferritin and haemosiderin. Ferritin consists of a core of up to 4500 iron atoms, enclosed in a large hollow protein shell [2]. This shell has a molecular mass of approx. 450000 [3] and is composed of heavy and light subunits, the relative amount of each depending on the tissue source of the ferritin. For example, ferritins from the heart and pancreas have a predominance of heavy subunits, whereas those from the liver and spleen have a predominance of light subunits [4,5].

Despite the essential nature of iron, it is toxic when present in excess. This toxicity may be due to the formation of the extremely reactive hydroxyl radical, following the interaction of iron with hydrogen peroxide [6]. Such interaction could be exacerbated by the superoxide radical, which has been shown to mobilize iron from horse spleen ferritin [7,8].

The ability of different tissues to tolerate excess iron is variable. Thus while an iron concentration of 2.5 mg/g in human liver is associated with tissue damage [9], the presence of more than four times this amount of iron in the liver of adult lampreys does not produce any evidence of damage [10]. Although the biological consequences of hydroxyl radical-mediated damage are dependent on the site of generation of this radical [11], a key step in the initiation of such damage is the mobilization of iron from storage sites such as ferritin. It is proposed that a potential mechanism for minimizing the effects of excess iron would be to restrict its release from ferritin.

The above hypothesis has been tested by determining whether the rate of iron release from ferritin is greater in tissues that have low iron levels and are susceptible to damage by excess iron, than in healthy tissues which typically contain high concentrations of iron. Comparisons have thus been made between the release of iron from the ferritins of rat, human and trout liver and those of lamprey liver and horse spleen.

MATERIALS AND METHODS

Chemicals

Ammonium ferrous sulphate was obtained from Ajax Chemicals (Sydney, Australia). Ammonium acetate, Hepes, hydrogen peroxide (30 %, w/v) and thiourea came from BDH (Port Fairy, Victoria, Australia). Bio-Rad (Richmond, CA, U.S.A.) supplied the Bradford reagent concentrate for the protein assay and also the Chelex 100 resin. Xanthine oxidase was purchased from Boehringer–Mannheim, Australia, or prepared from fresh cream as described below. Coomassie Blue R250 dye was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Sephacryl S300 and Sephadex G25 resins were obtained from Pharmacia (Upsala, Sweden). Bathophenanthroline disulphonic acid, BSA (fraction V powder), horse heart cytochrome *c* (type VI), Ferrozine, horse spleen ferritin (type 1), phenylmethanesulphonyl fluoride (PMSF), bovine erythrocyte superoxide dismutase (SOD), trichloroacetic acid, uric acid and xanthine (grade V) were all obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Tissue source

Human liver tissue was obtained post mortem from the Queen Elizabeth II Medical Centre from an accident victim showing no signs of any iron disorder. Livers were removed from *Rattus norvegicus* (strain PVG/c) provided by the Animal Resource Centre (Perth, Western Australia), the rainbow trout, *Oncorhynchus mykiss*, bred at Lesmurdie Trout Farm (Perth, Western Australia), and adults of the lamprey, *Geotria australis*, collected during their upstream migration in the Donnelly River, Western Australia (34 °S, 116 °E). Horse spleen was a gift from Murdoch University Veterinary Hospital. All tissues were stored frozen before use.

Preparation of ferritins

Ferritins were prepared from their respective tissues as follows:

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; SOD, superoxide dismutase; BBS, 25 mM borate-buffered physiological saline, pH 8.6.

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the tissue was minced, then homogenized in 25 mM borate-buffered physiological saline, pH 8.6 (BBS) containing 0.12 mM PMSF, at a temperature of 4 °C, using a Polytron homogenizer (Kinematica GmbH, Luzern, Switzerland). The homogenate was then centrifuged at 6000 *g* for 20 min at 4 °C in a Sorvall centrifuge. The resulting supernatant was filtered through three layers of cheesecloth, then transferred to a waterbath maintained at 75 °C. The supernatant was mixed periodically, allowed to reach 73 °C and left a further 10 min before being plunged into an ice slurry for 10 min. Subsequent purification steps were conducted at 4 °C.

Coagulated protein was separated from the ferritin-rich aqueous layer by centrifugation at 9000 *g*, and the supernatant was again filtered through three layers of cheesecloth. Due to heavy contamination by microsomes, liver samples were centrifuged on an exponential sucrose gradient (0.4–2.55 M sucrose [12]) for 6 h at 100000 *g* in a Beckman L8 ultracentrifuge using a 70 Ti rotor. After a 1:10 dilution with BBS, the ferritin-containing fractions of the supernatant were concentrated in an Amicon ultrafiltration cell using a PM30 membrane, before all ferritins were passed down a Sephacryl S300 column (90 cm × 2.5 cm) equilibrated with BBS. Fractions were collected using a Foxy fraction collector (ISCO, Lincoln, NE, U.S.A.), and the brown ferritin-containing fractions were pooled and concentrated as above. Preparative-scale electrophoresis was then performed on the concentrate, using a discontinuous native PAGE system (Protean: Bio-Rad, CA, U.S.A.) with a 5% polyacrylamide resolving gel, pH 8.8 [13]. Gels were run at 50 mA for 5 h at 10 °C. After electrophoresis, the clearly visible monomeric ferritin band was cut out and minced with a razor. The resulting gel fragments were then homogenized for 1–2 s in a 20–50-fold excess of BBS and the slurry was left overnight before centrifugation at 3000 *g* for 10 min in a Beckman GPR refrigerated centrifuge. The resulting supernatant containing the purified ferritin was concentrated as described previously and stored in the presence of 0.02% sodium azide. In order to ensure no microbial contamination of the proteins had occurred, samples of ferritins were plated on nutrient agar and incubated for 5 days at 23 °C. Immediately before use, all ferritins were stripped of surface-bound iron by passage through a 5 ml column of Chelex 100 resin. Dilution of the ferritins was compensated for by measuring the absorbance of the ferritin samples at 460 nm before and after passage down the Chelex 100 column, and multiplying the original ferritin iron content by the ratio of the two absorbances.

The protein content of the ferritins was determined by the method of Bradford [14], using BSA as a standard. The iron content of the various ferritins was determined by atomic absorption spectrometry, using a Varian model AA 175 spectrometer, or by the Ferrozine colorimetric iron assay of Yee and Goodwin [15].

Ferritin subunit composition was determined using discontinuous SDS/PAGE in a 15% acrylamide resolving gel [13] for 1 h at 150 V on a Bio-Rad Mini-Protean electrophoresis system. Samples were prepared by boiling for 10 min in 3% (w/v) SDS and 3% (v/v) 2-mecaptoethanol. After electrophoresis was complete the gels were stained with Coomassie Blue R250 [13].

In order to check for the release of free iron from ferritins during storage, ferritin samples were centrifuged at 160000 *g* (200 kPa) in a Beckman Airfuge for 35 min to pellet the ferritin, and an iron assay was performed on the supernatant as above. Negligible free iron was evident in the buffer in which the ferritin was stored, indicating that iron is not significantly mobilized from ferritin when a chelator is absent.

Xanthine oxidase preparation

Xanthine oxidase was prepared from fresh unpasteurized bovine cream, using the method of Rajagopalan [16]. The enzyme was then quickly frozen in liquid nitrogen and stored at −20 °C until use. On the day of assay, the enzyme preparation was passed down a 5 ml column of Sephadex G25 equilibrated with 10 mM Hepes buffer, pH 7, in order to remove traces of EDTA and salicylic acid which had been added during the isolation procedure in order to stabilize the enzyme. The xanthine oxidase was assayed using the method of Terada et al. [17], with one unit being that amount of enzyme which catalysed the formation of one μmol of uric acid per min at 25 °C, pH 7.0.

Characterization of the iron release assay

The superoxide ion was generated using a system consisting of 100 μM xanthine in 10 mM Hepes buffer, pH 7.0 at 25 °C, in a final volume of 1 ml, with the reaction being started by the addition of 0.02 unit of xanthine oxidase. All reagents used in the iron release component of this study were passed down a Chelex 100 column before use. Before using this assay system on ferritin, superoxide formation was monitored by including 60 μM cytochrome *c* in the superoxide-generating system and following its reduction at 550 nm, using a molar absorbance coefficient of $2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$ for reduced cytochrome *c*.

To demonstrate that no adventitious iron was present in the reagents used, xanthine, xanthine oxidase, Hepes buffer and bathophenanthroline were incubated together. Negligible iron contamination was present as there was no change in absorbance at 530 nm when compared with a bathophenanthroline–water blank. Xanthine and the end products of xanthine oxidase acting on xanthine, i.e. uric acid and hydrogen peroxide, were also tested for their ability to mobilize iron from ferritin in the absence of xanthine oxidase. Negligible effects were found at concentrations which could be generated by the enzyme system used in this study.

In order to measure iron release from the various ferritins, ferritin normalized to 100 μg of iron, 1 mM bathophenanthroline and, where appropriate, SOD (48 units) were incorporated into the superoxide-generating system. Before the addition of xanthine oxidase, the absorbance was monitored for 10 min to determine the basal release or iron from ferritin under these conditions. The assay was then initiated by the addition of the enzyme, raising the total volume to 1 ml, and the absorbance of the subsequent Fe(bathophenanthroline)$_3$ complex was monitored at 530 nm for an additional 10 min period. The basal release of iron was subtracted from the xanthine oxidase-mediated iron release and the initial rate of iron release was found by fitting a polynomial equation to the data and extracting the first-order coefficient. After assay, the reaction mixture containing ferritin, xanthine oxidase and SOD was subjected to non-dissociating discontinuous PAGE on a 5% gel [13].

RESULTS

Iron/protein ratios and ferritin subunit structure

The iron to protein ratios (w/w) for the ferritins used in this study ranged from 0.14 for Sigma horse spleen to 0.23 for trout liver (Table 1). When analysed by SDS/PAGE, all ferritins showed minor bands which appeared to be dimers and trimers of ferritin subunits. Subunit size ranged from 18500 for that of horse spleen ferritin to 20800 for that of trout liver ferritin (Table 1).

Table 1 Iron:protein ratios and subunit composition of ferritins used in this study

Abbreviation: n.d., not determined.

Ferritin	Iron:protein	Major subunit size (Da)
Human liver	0.17	20500
Rat liver	0.15	20000
Trout liver	0.23	20800
Lamprey liver	0.20	20300
Horse spleen	0.17	19300
Horse spleen (Sigma)	0.14	n.d.

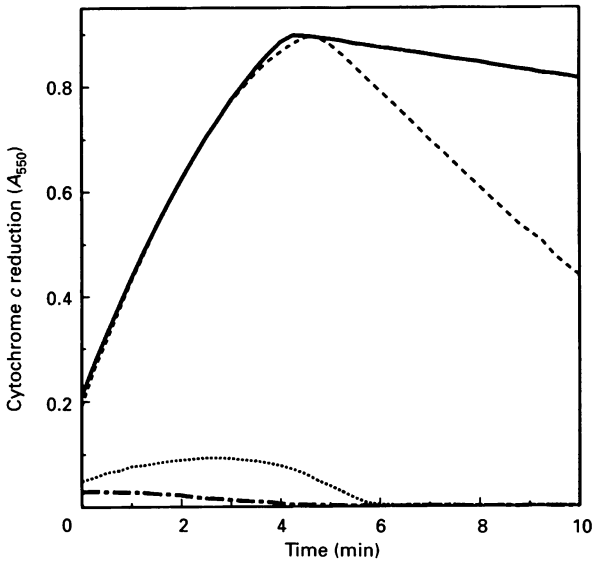

Figure 1 Validation of the superoxide-generating system

Xanthine oxidase, either prepared according to the method of Rajagopalan [16] (——) or purchased from Boehringer Mannheim (– – –), was tested for its ability to reduce cytochrome *c*. Superoxide-independent reduction of cytochrome *c* was assessed by the inclusion of excess of SOD with our preparation (— · —) and Boehringer–Mannheim's preparation (· · ·). Reaction mixtures contained 100 μM xanthine, 0.02 unit xanthine oxidase and 60 μM cytochrome *c* in 10 mM Hepes buffer, pH 7.0, with 48 units of SOD where appropriate.

Validation of the superoxide-generating system

Superoxide formation, as measured by cytochrome *c* reduction, was linear for the first minute, then became increasingly less linear and finally underwent an abrupt decline after 4.5 min (Figure 1). This pattern was mirrored by the accumulation of uric acid (results not shown). The original rate of superoxide production could be restored by a subsequent addition of xanthine, indicating substrate limitation and minimal product inhibition by uric acid at the concentrations likely to be encountered in this assay. The subsequent decline in absorbance (Figure 1), showed that Boehringer–Mannheim's xanthine oxidase preparation had a marked tendency to reoxidize the cytochrome *c* after the formation of superoxide had ceased. This reoxidation could be eliminated by the addition of catalase (results not shown), indicating that it was contaminated with lactoperoxidase, as has previously been noted [18]. This commercial source of xanthine oxidase also produced a significant

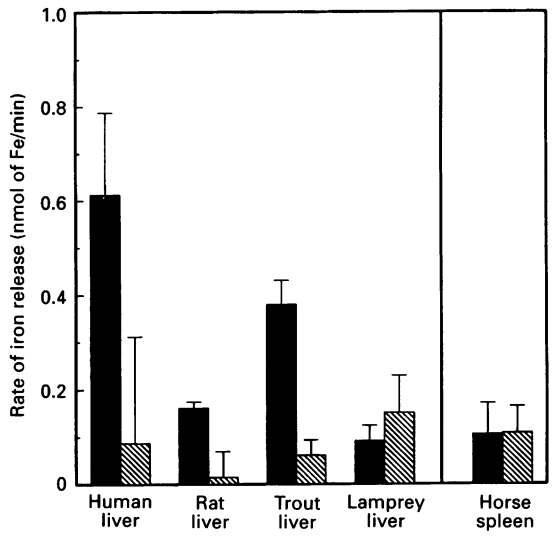

Figure 2 Release of iron from various ferritins

Ferritins from various species were incubated in the presence of xanthine oxidase and xanthine. The initial rate of total iron release (■) and its superoxide-independent component (▨), determined as described in the Materials and methods section, are shown. The basal release of iron to bathophenanthroline, which occurred in the absence of xanthine oxidase, has been subtracted from these results. Error bars represent S.E.M. values except for human and trout liver superoxide-independent release where they represent S.D.

superoxide-independent reduction of cytochrome *c* between 0 and 3 min, indicating damage to the enzyme [18]. In contrast, the rate of reoxidation of cytochrome *c* by our xanthine oxidase preparation, although still detectable, was much lower and, more importantly, there was no superoxide-independent reduction of cytochrome *c* (Figure 1). Because of this latter significant difference in the two preparations of xanthine oxidase, all subsequent experiments were conducted using our preparation.

Basal iron release

All ferritins showed a low but obvious iron release to bathophenanthroline in the absence of superoxide formation by xanthine oxidase. These values ranged from 0.05 nmol/min for human liver ferritin to 0.01 nmol/min for lamprey liver ferritin with the order of release being human liver > horse spleen > trout liver > rat liver > lamprey liver (results not shown). Since all of the ferritins had been stripped of surface-bound iron, this release must represent a loss of iron from the core or internal region of the protein shell. A commercial source of horse spleen ferritin (Sigma) showed a similar, but slightly higher, basal release of iron, indicating that this phenomenon was not restricted to ferritins prepared in our laboratory. To determine whether this basal release was sustained, horse spleen ferritin was incubated for an extended period (4 h), during which the rate of iron release remained constant (results not shown).

Iron release by xanthine oxidase in the presence and absence of SOD

The initial rate of iron release, in the presence of the superoxide-generating system, was higher for ferritins from human, trout and rat liver than for those from lamprey liver and horse spleen (Figure 2). In the case of human and trout ferritins, this difference was significant ($P < 0.05$). The addition of SOD to the ferritins

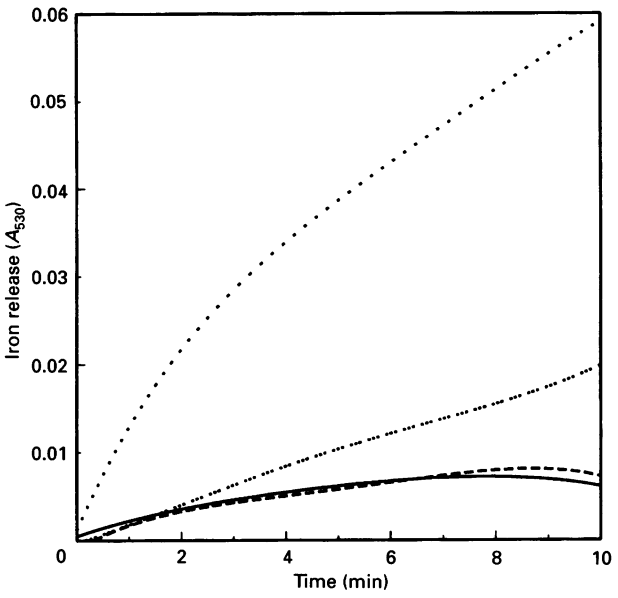

Figure 3 Release of iron from human and lamprey liver ferritins

Human liver ferritin (· · ·) and lamprey liver ferritin (——) were incubated in the presence of active xanthine oxidase, and the subsequent release of iron to bathophenanthroline was monitored. The superoxide-dependent component of this iron release was determined by the addition of excess SOD: (··· ···), human liver ferritin + SOD; (– – –), lamprey liver ferritin + SOD. The basal release of iron to bathophenanthroline, which occurred in the absence of xanthine oxidase, has been subtracted from these results.

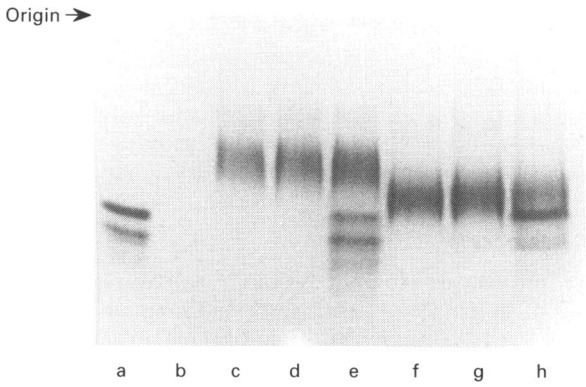

Figure 4 PAGE analysis of rat and lamprey liver ferritins before and after exposure to xanthine oxidase and SOD

Rat liver ferritin (susceptible to superoxide-dependent iron release) and lamprey liver ferritin (resistant to superoxide-dependent iron release) are shown here. Wells contain: (lane a), SOD; (lane b), xanthine oxidase; (lane c), rat liver ferritin; (lane d), rat liver ferritin after exposure to xanthine oxidase; (lane e), rat liver ferritin after exposure to xanthine oxidase and SOD; (lane f), lamprey liver ferritin; (lane g), lamprey liver ferritin after exposure to xanthine oxidase; and (lane h) lamprey liver ferritin after exposure to xanthine oxidase and SOD.

of human, rat and trout liver led in each case to a significantly lower initial rate of iron release ($P < 0.05$). In contrast, the release of iron from lamprey liver and horse spleen ferritins was unaffected by the addition of SOD (Figure 2). The contribution of superoxide-dependent release to total iron release from human, rat and trout liver ferritin was 86%, 92% and 84% respectively, whereas it did not contribute to the release of iron from either lamprey liver or horse spleen ferritin. An analysis of these latter two ferritins for superoxide dismutation activity (results not shown), indicated that the absence of a superoxide-dependent iron release was not due to them being contaminated with SOD.

In the case of human liver ferritin, the elevated rate of release of iron in the presence of the superoxide-generating system continued for the 10 min duration of the experiment (Figure 3). Although there was a downturn in the rate of iron release, it was still significantly elevated after 4.5 min, when the generation of the superoxide ion had ceased (see Figure 1). Throughout the whole of the experiment, the rate of iron release from human liver ferritin was significantly lowered by the addition of SOD. When SOD was present, the rate of iron release remained constant for the entire experiment (Figure 3). In the presence of the superoxide-generating system, the rate of iron release was significantly lower from both lamprey liver ferritin (Figure 3) and horse spleen ferritin (results not shown), than from human liver ferritin. In contrast with the marked decrease in the rate of iron release from human liver ferritin following the addition of SOD, the addition of this enzyme had no effect on iron release from lamprey liver ferritin.

In order to ascertain whether the addition of the superoxide-generating system and SOD affected the physical properties of the ferritins, each ferritin was analysed by PAGE before and after the above additions. Figure 4 demonstrates that for rat and lamprey liver ferritins, the migration of the ferritin was unaffected by these additions. Similar results were also obtained with human and trout liver ferritins and horse spleen ferritin (results not shown).

DISCUSSION

Each of the ferritins investigated in this study release iron very slowly in the presence of the chelator bathophenanthroline. This is in agreement with previous observations of iron release from various ferritins with other chelators [19–21]. The presence of active xanthine oxidase significantly elevated the rate of iron release from each of the ferritins examined. This additional iron release by xanthine oxidase occurs via two distinct mechanisms, one of which is superoxide-dependent and the other superoxide-independent (Figures 2 and 3). The relative contribution of these two processes differed, however, among the various ferritins (Figure 2). Ferritins isolated from organs that contain high levels of iron, e.g. lamprey liver and horse spleen [10,22], show negligible superoxide-mediated iron release. In contrast, ferritins isolated from human, trout and rat liver, which contain low levels of iron [23–25], release iron predominantly by a superoxide-mediated mechanism. This suggests that a lack of tissue damage under high iron loading, a situation which occurs in lamprey tissues and horse spleen, may in part be due to the ability of ferritins from these tissues to resist superoxide-mediated iron release. However, caution must be taken before concluding that similar differences occur under physiological conditions. The elevated rate of release of iron from human, rat and trout liver ferritins may only occur in the presence of superoxide ions when a high-affinity chelator, such as bathophenanthroline, is also present. Alternatively, under cellular conditions, the rate of superoxide generation, or the activity of SOD, may be such that rates of iron release are not enhanced. Nevertheless, the data suggest that ferritins from iron-sensitive tissues may be more likely to release their iron than those from iron-resistant tissues.

The differences in the rates of iron release from the various ferritins did not correlate with either the subunit size, or the iron:protein ratios of these ferritins (Table 1). Furthermore, as there was negligible superoxide dismutation activity associated with either horse or lamprey ferritin, the insensitivity of these

ferritins to iron release by superoxide cannot have resulted from them being contaminated with SOD. It is also of note that the protein shells of each of the ferritins were apparently unaffected by the addition of both the superoxide-generating system and SOD, as is apparent from their identical rate of migration when subjected to PAGE before and after the above additions (Figure 4).

Our finding, that superoxide ions have no effect on iron release from horse spleen ferritin, differs from previous reports, which showed that approx. 70% of the xanthine oxidase-mediated iron release from horse spleen ferritin was due to superoxide ions [7,8,26]. In addition, these reported rates of iron release were higher than that presented here. These differences are probably artefacts of the purification process used to isolate these ferritins. In contrast with our preparations, the ferritins supplied by both Sigma and Boehringer and used by the above authors, are prepared by precipitation with ammonium sulphate. Measurements in this laboratory (results not shown) have demonstrated that the pH of both Sigma and Boehringer horse spleen ferritin is approx. 5.5, whereas that of our preparation was 7.0. It has been shown that acidic pH accelerates the release of iron from ferritin [21,27,28], and prolonged storage at pH 5.5 could possibly enhance the rate of iron release from ferritin by altering the structure of the iron core. When we used Sigma horse spleen ferritin, we obtained results comparable with those of Bolann and Ulvik [7] and Biemond et al. [26], both in terms of the rate of iron release and the proportion which was superoxide-dependent. It is thus likely that horse spleen ferritin retains its iron more strongly in the presence of either a superoxide-generating system or a chelator than has previously been suggested [7,19,26].

For each of the ferritins studied, a significant component of iron release due to xanthine oxidase is superoxide-independent (Figure 2). This iron release is additional to that which occurs in the presence of the chelator alone, indicating that xanthine oxidase is also capable of releasing iron from ferritin by a mechanism not involving superoxide ions. This occurs despite the fact that the xanthine oxidase used in this study was isolated so as to minimize any damage to the enzyme which can occur during purification [18], and thus eliminate the superoxide-independent reduction of cytochrome *c* that is associated with some commercial preparations.

The data presented in this paper clearly demonstrate that the kinetics of iron release from various vertebrate ferritins differ when these ferritins are exposed to a superoxide-generating system. This may be due to differences in the structure of their iron cores. While there is now general agreement that the core is composed of ferrihydrite with a semi-random structure [29], different ferritins range from amorphous to highly crystalline, depending on their origin and mode of formation [29–31]. Adult lamprey liver ferritin is highly crystalline, with only 10% being in a non-crystalline phase [32], and this may account for its relatively low rate of iron release. In contrast, human and rat liver ferritins, which are more susceptible to superoxide-mediated iron release, are more amorphous in structure [1].

It has already been established that the lamprey, which has the ability to tolerate the highest known levels of iron in any vertebrate [10] uses several means of minimizing the potentially toxic effects of such high iron loading. Thus, with increasing iron load, a greater proportion of iron is compartmentalized into the less accessible storage protein, haemosiderin [10,33,34], and the activity of SOD rises proportionately [25,35]. This study demonstrates that, in addition to these mechanisms, the lamprey may be further protected by having a ferritin that is particularly resistant to superoxide-mediated iron release.

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