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AN IRON CHELATOR IS NOT REQUIRED FOR REDUCTIVE IRON RELEASE FROM FERRITIN BY RADICAL GENERATING SYSTEMS

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Although a number of reducing systems can release iron from ferritin, there is debate as to whether the process additionally requires a chelator. We have studied ferritin iron release by microsomes, paraquat and NADPH, by dialuric acid and by hypoxanthine and xanthine oxidase, using ferrozine to complex the released iron. In each case, Fe^{2+}(ferrozine) formation was detectable when the ferrozine was added at the beginning of the 10 min reaction period, but not at the end. However, with catalase present, up to 0.7 times as much Fe^{2+} could be measured with ferrozine added at the end. Further Fe^{2+} could be recovered by adding ascorbate with the ferrozine. These results indicate that an iron chelator is not required for reductive iron release from ferritin. However, the released iron will not be detectable as Fe^{2+} unless it forms a complex that is resistant to oxidation by H_2O_2 or other oxidants.

KEY WORDS: Ferritin iron release, ferrozine

INTRODUCTION

Ferritin stores approximately 20% of all iron in mammals as a ferric oxyhydroxide polymer. Reductive release of iron from ferritin was first demonstrated in 1955.[1] With the subsequent demonstration that O_2^- and a number of biologically relevant xenobiotic radicals can release ferritin-iron,[2-5] considerable interest has focused on ferritin as a physiological source of iron for catalysing free radical reactions. In a recent report, Reif *et al.*,[6] observed that in order to detect iron release from ferritin by radiolytically generated O_2^-, it was necessary to have an Fe^{2+} chelator (bathophenanthroline) present during irradiation. Adding bathophenanthroline after irradiation yielded no Fe^{2+}-complex. These results led them to propose that radical-mediated iron mobilization from ferritin requires an iron chelator. However, an alternative explanation of their results is that Fe^{2+} could have been released, but subsequently oxidized to Fe^{3+}, which would give a colourless complex on addition of bathophenanthroline. The present study examines the chelator requirement for iron release by determining whether oxidants formed during the process could prevent detection of released Fe^{2+}. The effects of catalase and ascorbate on the recovery of Fe^{2+} were examined, using three different methods of releasing ferritin iron.

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MATERIALS AND METHODS

Ferritin (from horse spleen) containing 1.5 nmol iron/μg as measured by the method of Hoy *et al.*,[7] ferrozine, paraquat, NADPH, hypoxanthine, xanthine oxidase, and catalase were purchased from Sigma Chemical Co, St Louis, MO. Catalase was not contaminated with SOD, as determined in a cytochrome c reduction assay. Dialuric acid was synthesized[8] and kindly supplied by Dr Rex Munday (Ruakura Animal Centre, Hamilton, NZ). All other reagents were of analytical grade from BDH, Poole, UK. Solutions were prepared in deionized water (Milli Q System) and buffers were stirred with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) to decrease contamination by transition metals. Xanthine oxidase was further purified as described.[9] Liver microsomes were prepared from Sprague-Dawley rats.[10]

Iron release from ferritin (200 μg/ml) was carried out in phosphate-buffered saline, pH 7.4, at 22°C under aerobic conditions. It was determined as an increase in A_{562} of solutions (1 ml total volume) containing ferrozine (200 μM; $\varepsilon_{562} = 27{,}900\,M^{-1}cm^{-1}$)[11] present from the beginning of the 10 min incubation in the continuous assay, or with ferrozine added after 10 min in the discontinuous assay. Iron release systems contained (i) rat liver microsomes (0.5 mg/ml), NADPH (100 μM) and paraquat (5 μM),[10] (ii) dialuric acid (100 μM)[12] and (iii) hypoxanthine (50 μM) and xanthine oxidase (0.01 U/ml).[9]

RESULTS AND DISCUSSION

Three systems that we have studied previously were used to release iron from ferritin. The first consisted of microsomes incubated with paraquat and NADPH in air.[10] This system gives maximal iron release under N_2 where paraquat radicals are responsible. In air, the reaction is partially inhibited because some of the paraquat radicals react with O_2 to give O_2^-, and both radicals contribute to the iron release. The second system was dialuric acid in neutral buffer under air. The dialuric acid undergoes rapid autoxidation generating O_2^- and H_2O_2, but it is the dialuric acid itself that is primarily responsible for iron release.[12] The third system consisted of hypoxanthine and xanthine oxidase under air,[9] in which case iron release is due to O_2^-.[3,9]

As observed previously, all three systems released iron from ferritin at an appreciable rate, when ferrozine was present throughout the incubation period (Table 1). There was no detectable increase in Fe^{2+}(ferrozine) absorbance in the absence of added ferritin, indicating that iron release from endogenous microsomal ferritin[13] or other non-ferritin iron was negligible in all systems. Rates of Fe^{2+}(ferrozine) accumulation were enhanced slightly by including catalase to remove H_2O_2 generated during the reactions. When ferrozine was added at the end of the incubation period (discontinuous assay) no Fe^{2+}-complex was detected (Table I), in agreement with the results of Reif *et al.*[6] However, adding catalase to the microsomal system or to dialuric acid enabled approximately 70% of the iron release measured by the continuous assay to be detected with ferrozine added at the end of the incubation. With the xanthine oxidase system in the presence of catalase, only a quarter as much iron release was detected by the discontinuous assay, but the remainder could be recovered as Fe^{2+} (ferrozine) on adding ascorbate (Table I). Addition of ascorbate to ferritin and ferrozine alone gave no detectable iron release over this time period, indicating that the ascorbate was reducing released iron.

These results show that iron was released from ferritin in the presence and absence of ferrozine. A small fraction of the released iron was oxidized by H_2O_2 when

TABLE I
Effect of catalase on a continuous and discontinuous assay of iron released from ferritin by 3 reducing systems[a]

System		Fe^{2+} released in 10 min (μM)			
		Continuous		Discontinuous	
		− Catalase	+ Catalase	− Catalase	+ Catalase
i.	Microsomes/paraquat/NADPH	0.72	0.86	0.00	0.58
i.	Dialuric acid	0.64	1.07	0.00	0.79
iii.	Hypoxanthine/xanthine oxidase	0.68	0.82	0.03	0.21
	Hypoxanthine/xanthine oxidase. Ascorbate added after 10 min reaction	N.D.	0.84	0.75	0.81

[a] Reaction conditions are given in the Methods section. Catalase (60 μg/ml) and ascorbate (3 mM) were added where indicated. Results are means of triplicates which agreed within 10%. N.D. = Not determined.

ferrozine was present, but in the absence of a specific chelator all the released iron was oxidized and not detectable as a Fe^{2+}-complex. Most of the oxidation was due to H_2O_2, but particularly with the xanthine oxidase system, the inability of catalase to prevent oxidation implies a contribution by other oxidants. These results are compatible with the known stability of ferrous bipyridyl complexes to oxidation. They are consistent with the observation[9] that the iron released from ferritin with xanthine oxidase-derived O_2^-, in the absence of a specific chelator, is recovered in the low molecular weight fraction on gel filtration. They also suggest that iron release was not detected previously in the absence of added chelator[6] because it was oxidized by the H_2O_2 generated during radiolysis.

We conclude, therefore, that an iron chelator is not required for reductive iron release from ferritin. However, unless the released Fe^{2+} forms a complex, e.g. with a bipyridyl, that is resistant to oxidation, then it will not be detected as Fe^{2+}.

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