Reductive Release of Iron from Ferritin by Cation Free Radicals of Paraquat and Other Bipyridyls*

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NADPH-cytochrome P-450 reductase-catalyzed reduction of paraquat promoted the release of iron from ferritin. Aerobically, iron release was inhibited approximately 60% by superoxide dismutase, whereas xanthine oxidase-dependent iron release was inhibited nearly 100%. This suggests that both superoxide and the paraquat cation radical can catalyze the release of iron from ferritin. Accordingly, under anaerobic conditions, the paraquat radical mediated a very rapid, complete release of iron from ferritin. Similarly, the cation free radicals of the closely related chemicals, diquat and benzyli viologen, also promoted iron release. ESR studies demonstrated that electron transfer from the paraquat cation radical to ferritin accounts for the reductive release of iron. The ferritin structure was not altered by exposure to the paraquat radical and also retained its ability to reincorporate iron. These studies indicate that release of iron from ferritin may be a common feature contributing to free radical-mediated toxicities.

The toxicity associated with numerous drugs and chemicals is often attributed to their ability to be metabolized to free radical species. The free radicals of many of these compounds, such as paraquat and related bipyridyls, undergo cyclic reduction and autoxidation resulting in the production of partially reduced forms of dioxygen such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (1). Many studies have linked excessive O$_2^-$ and/or H$_2$O$_2$ production with oxidative damage to numerous biological macromolecules including lipids, proteins, and DNA (2). However, neither O$_2^-$ nor H$_2$O$_2$ is considered to be of sufficient reactivity to directly initiate and/or promote oxidation of cellular constituents (3). Therefore, oxidants capable of initiating lipid peroxidation and other deleterious oxidative processes are generally proposed to result from reactions between these species and transition metals (4).

Although the initiating species has not been clearly identified, numerous studies indicate that iron can be intimately involved in the peroxidative process. Thus, the in vivo formation of an initiator is likely to be contingent upon the availability of physiological iron. Although iron metabolism has been the subject of considerable study, the possibility that iron may participate in these deleterious oxidations in vivo remains largely unexplored. At present, the most likely catalyst for mediating oxidative processes is thought to be a low molecular weight intermediate functioning to transport iron between transferrin and ferritin or between ferritin and other iron-requiring biochemical processes (5). Some evidence for such complex(es) in erythrocytes has been presented (6, 7), although in other tissues such as liver a variety of potential chelators such as ADP (8) or citrate (9) have been proposed but not conclusively identified.

While the existence of low molecular weight iron chelates remains speculative, it is clear that the vast majority of iron, other than heme iron as in hemoglobin, is present in the high molecular weight proteins transferrin, ferritin, and hemosiderin (10). The relative insolubility of ferric iron and its potential toxicity necessitates its complexation by these proteins, so it is likely that release of iron from the protein(s) is necessary for catalysis of the aforementioned reactions. Most of the intracellular iron is stored within ferritin, a large spherical protein consisting of 24 subunits arranged symmetrically around a hollow core in which ferric iron is held in crystalline aggregates (11). Access to the central core is via six narrow, hydrophobic channels through which the iron can be shuttled.

The release of ferric iron from ferritin appears to require reduction (12); however, positive identification of the physiological reductant(s) has not been achieved. Harrison and colleagues (13) suggest that reductants pass through the channels and interact directly with the ferric hydroxide core, whereas others envision the existence of oxidoreduction sites on the interior of the channels (14). It has been demonstrated that reduced flavins, which are among the most potent reductants for releasing ferritin iron, must at least partially traverse the channels in order to release iron (15, 16). Furthermore, reductive mobilization of iron from ferritin appears to be dependent upon formation of a complex at the surface of the iron hydroxide core by the reductant (16). Thus, biological agents capable of releasing iron from ferritin may be limited by their size as well as their potential to form complexes. Another factor that must be taken into consideration is the redox potential of the reductant, as recent work reports the reduction potential for ferritin iron to be approximately -230 mV at pH 7.0 (17).

We have previously demonstrated that O$_2^-$ generated by xanthine oxidase, can reductively release iron from ferritin and promote peroxidation of phospholipid liposomes (18). Superoxide-dependent iron release from ferritin by stimulated polymorphonuclear leukocytes has also been demonstrated (19). Subsequent studies demonstrated that the redox cycling of paraquat promoted ferritin-dependent lipid peroxidation (20). These results support the reports which indicate that paraquat-induced lipid peroxidation is a result of enhanced cellular O$_2^-$ production (21, 22). However, exposure of Esche-
Effects of Superoxide Dismutase and O_2 on Iron Release from Ferritin—The results in Table I demonstrate that the release of iron from ferritin by NADPH-cytochrome P-450 reductase and paraquat is only inhibited 82% by superoxide dismutase. This is in contrast to the 97% inhibition observed when superoxide dismutase is added to a system containing xanthine and xanthine oxidase. A low rate of iron release was observed with NADPH-cytochrome P-450 reductase in the absence of paraquat, and this activity was almost completely inhibited by the addition of superoxide dismutase. The high inhibition observed in the presence of superoxide dismutase confirms the involvement of free radicals in the iron release process.

The abbreviation used is: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
inhibited by the addition of superoxide dismutase. The data in Table II demonstrate that the rate of iron release from ferritin isolated from the livers of control animals (native) is much greater than that from ferritin obtained from animals pretreated with iron-dextran (iron-induced). However, it can also be seen that, even when the ferritin concentration was varied greatly, paraquat-dependent iron release from both ferritin preparations was consistently inhibited only 50–60% by superoxide dismutase.

Effect of Varying Concentrations of Ferritin, NADPH-Cytochrome P-450 Reductase, and Paraquat on Iron Release from Ferritin by Paraquat—The quantity of iron released by paraquat anaerobically was assessed by continuously monitoring iron release from ferritin until no further formation of the bathophenanthroline sulfonate-ferrous complex was observed. Results are the averages of two separate experiments.

In Fig. 1 it can be seen that when NADPH-cytochrome P-450 reductase and paraquat were incubated with purified rat liver ferritin, a lag period of approximately 7 min was observed before a rapid release of iron from ferritin occurred, as indicated by formation of the Fe2+—bathophenanthroline complex absorbing at 530 nm (curve A). When the reaction mixture was purged with argon, to decrease the O2 content prior to the addition of NADPH, the lag period was shortened to approximately 3 min (curve B). When glucose oxidase, glucose, and catalase were included to further reduce the concentration of O2, iron release was observed immediately (curve C).

Effect of Varying Concentrations of Ferritin, NADPH-Cytochrome P-450 Reductase, and Paraquat on Iron Release from Ferritin by Paraquat—The quantity of iron released by paraquat anaerobically was assessed by continuously monitoring iron release from ferritin until no further formation of the bathophenanthroline sulfonate-ferrous complex was observed. The data in Table III demonstrate that all of the iron was released from ferritin over a range of ferritin concentrations when NADPH-cytochrome P-450 reductase and paraquat were incubated with ferritin anaerobically. As shown in Fig. 2, the time required to release all of the iron from ferritin was decreased by increasing NADPH-cytochrome P-450 reductase activity. A marked decrease in the amount of time required for complete release of iron was also observed when the concentration of paraquat was increased, up to approximately 0.10 mM paraquat (Fig. 3).

**Table I**

**Effect of superoxide dismutase on xanthine oxidase and paraquat-dependent release of iron from ferritin**

| System                  | +Superoxide dismutase | −Superoxide dismutase |
|-------------------------|-----------------------|-----------------------|
| Xanthine oxidase        | 0.64                  | 0.02                  |
| NADPH-cytochrome P-450  | 0.19                  | 0.03                  |
| Native ferritin         |                       |                       |
| 0.1 mg (94)             | 0.21                  | 0.04                  |
| 0.25 mg (235)           | 0.42                  | 0.07                  |
| 0.50 mg (470)           | 0.80                  | 0.16                  |
| 1.00 mg (940)           | 1.54                  | 0.38                  |
| 1.50 mg (1410)          | 1.76                  | 0.54                  |
| Iron-induced ferritin   |                       |                       |
| 0.1 mg (320)            | 0.12                  | 0.05                  |
| 0.25 mg (800)           | 0.38                  | 0.14                  |
| 0.50 mg (1600)          | 0.74                  | 0.29                  |
| 1.00 mg (3200)          | 0.91                  | 0.45                  |
| 1.50 mg (4800)          | 1.44                  | 0.72                  |

**Table II**

**Effect of superoxide dismutase on aerobic, paraquat-dependent release of iron from ferritin**

| Ferritin (nmol Fe++) | −Superoxide dismutase | +Superoxide dismutase |
|----------------------|-----------------------|-----------------------|
| Native ferritin      |                       |                       |
| 0.1 mg               | 0.21                  | 0.04                  |
| 0.25 mg              | 0.42                  | 0.07                  |
| 0.50 mg              | 0.80                  | 0.16                  |
| 1.00 mg              | 1.54                  | 0.38                  |
| 1.50 mg              | 1.76                  | 0.54                  |
| Iron-induced ferritin|                       |                       |
| 0.1 mg               | 0.12                  | 0.05                  |
| 0.25 mg              | 0.38                  | 0.14                  |
| 0.50 mg              | 0.74                  | 0.29                  |
| 1.00 mg              | 0.91                  | 0.45                  |
| 1.50 mg              | 1.44                  | 0.72                  |

**Table III**

**Anaerobic iron release from ferritin by paraquat at varying ferritin concentrations**

| Ferritin Fe2+ added | Total Fe3+ reduced |
|---------------------|-------------------|
| 0                   | 0.21              |
| 25                  | 26.1              |
| 50                  | 50.4              |
| 75                  | 74.9              |
| 100                 | 96.1              |
nmol of NADPH were oxidized per nmol of iron released for the three chemicals tested.

**Effect of Ferritin on Detection of the Paraquat Radical**—As shown in Fig. 4, as little as 8 µg/ml ferritin (25 µM Fe⁴⁺) reduced the 603-nm absorbance of the paraquat radical (curve B). When the amount of ferritin was continually increased, up to 64 µg/ml protein containing 200 µM Fe⁴⁺ (curves C–E), a lag period preceding detection of the radical was observed.

**TABLE IV**

| Compound  | Iron released | NADPH oxidation | NADPH oxidized/iron released |
|-----------|---------------|-----------------|-----------------------------|
|           | nml/min       | nmol            |                             |
| Paraquat  | 4.88          | 11.58           | 2.37                        |
| Diquat    | 5.05          | 12.22           | 2.42                        |
| Benzyl viologen | 3.43 | 9.00           | 2.62                        |

Reaction mixtures for iron release (1 ml final volume) contained NADPH-cytochrome P-450 reductase (0.1 unit), rat liver ferritin (25 µM Fe⁴⁺), bathophenanthroline sulfonate (1 mM), catalase (1000 units), glucose (5 mM), glucose oxidase (10 units), and varying amounts of NADPH-cytochrome P-450 reductase as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continuously monitored at 530 nm.

Following this lag, the paraquat radical then began to gradually accumulate, as evidenced by an increase in absorbance at 603 nm.

Reduction of paraquat to its cation radical by NADPH-cytochrome P-450 reductase under anaerobic conditions was also confirmed by ESR spectroscopy as seen in Fig. 5 (spectrum A). The addition of increasing amounts of ferritin (spectra B–E) resulted in a progressive quenching of the ESR free radical signal such that no signal was observed initially at concentrations of ferritin greater than 32 µg/ml (100 µM Fe⁴⁺).

**Structure and Iron Uptake Ability of Ferritin following Iron Release**—In Fig. 6 is shown the uptake of ferrous iron by ferritin, which had been incubated with NADPH-cytochrome P-450 reductase alone or with paraquat under aerobic and anaerobic conditions. The ferritin preparations from which the iron was released by the paraquat radical under anaerobic conditions, and that subjected to chemical reduction by dithionite, exhibited similar rates of iron uptake which were also the most rapid. The slowest rate of iron uptake was observed with ferritin that had not been exposed to an iron release system, whereas the ferritin preparations incubated aerobically with NADPH-cytochrome P-450 reductase, in the presence and absence of paraquat, demonstrated intermediate rates of iron uptake.
Free Radical-mediated Ferritin Iron Release

Fig. 5. Effect of ferritin on detection of the paraquat cation radical by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P-450 reductase (0.1 unit), catalase (500 units), paraquat (0.25 mM), glucose (5 mM), and glucose oxidase (10 units) in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and scanned immediately with ESR conditions as outlined under "Materials and Methods." Rat liver ferritin was included at the following concentrations: A, no additions; B, 8 µg (25 µM Fe²⁺); C, 16 µg (50 µM Fe²⁺); D, 32 µg (100 µM Fe²⁺); and E, 64 µg (200 µM Fe²⁺).

Fig. 6. Iron uptake by ferritin. Reaction mixtures (5 ml final volume) contained ferritin (90 µg/ml) in 0.1 M Hepes, pH 6.8. Reactions were started by the addition of ferrous ammonium sulfate (0.5 mM) and quenched in 0.3 g of Chelex 100 as described under "Materials and Methods." The absorbance at 310 nm was recorded as an index of iron uptake. The ferritin preparations were recovered from ferrous iron within ferritin appears to compete effectively with oxygen for the radical as evidenced by the ability of the radical to release nearly 50% of the iron from ferritin even under aerobic conditions.

Similarly reaction mixtures were also subjected to both native and sodium dodecyl sulfate-gel electrophoresis. The assembled ferritin protein did not appear to be affected by exposure to the radical when compared to the nontreated ferritin sample. Likewise, the individual subunits exhibited mobility identical to that of the nontreated ferritin, and no subunit fragments of less than approximately M, 18,000 were detected (results not shown).

DISCUSSION

The results of this study demonstrate that free radicals produced by NADPH-cytochrome P-450 reductase-catalyzed reduction are capable of releasing iron from ferritin. The toxicity associated with these compounds is often attributed to their ability to undergo redox cycling resulting in the production of active oxygen species leading ultimately to oxidative damage of cellular constituents, including membrane unsaturated lipids, proteins, and DNA (2). In general, it appears that oxidative damage requires, or is greatly potentiated by, the presence of transition metals; therefore, the identification of biological sources of iron is critical to an understanding of the mechanism(s) governing the toxicity of these chemicals. We had previously demonstrated the ability of O₂⁻ generated by xanthine oxidase, to release iron from ferritin (18), suggesting that increased production of O₂⁻ in biological systems may contribute to oxidative stress by increasing the amount of iron available to participate in redox reactions. Similarly, the redox cycling of paraquat promoted ferritin-dependent lipid peroxidation (20).

Xanthine oxidase-dependent iron release was inhibited 97% by superoxide dismutase, showing that the enzyme can prevent essentially all of the O₂⁻-dependent iron release. However, the present data demonstrate that under aerobic conditions superoxide dismutase only partially prevents paraquat-dependent release of iron from ferritin. Iron release from ferritin isolated from animals not pretreated with iron was even greater, as has been previously demonstrated using reduced flavins (41). This suggests that iron release from ferritin may be even more significant in vivo. The difference between total iron release and superoxide dismutase-sensitive iron release was used to assess the O₂⁻-independent release of iron and indicated that 40–50% of the iron released could be attributed to reduction by a reductant other than O₂⁻. The direct reduction of Fe³⁺ by the paraquat radical has been previously reported (27, 28). Recent work has indicated that the reduction potential required for ferritin iron release is approximately −230 mV at pH 7.0 (17); therefore, the paraquat radical (−444 mV) (39) may be more effective at mediating the reductive release of iron from ferritin than O₂⁻ (−330 mV) (42). The second order rate constant for the reaction of the paraquat radical with oxygen is 8 × 10² M⁻¹ s⁻¹ (43) and 2.1 × 10³ M⁻¹ s⁻¹ with cytochrome c (28). In these studies, the ferric iron within ferritin appears to compete effectively with oxygen for the radical as evidenced by the ability of the radical to release nearly 50% of the iron from ferritin even under aerobic conditions.

In agreement with the above data, the results in Fig. 1 demonstrate the efficiency with which the paraquat radical can release ferritin iron. Under aerobic conditions, a very low rate of iron release occurs. However, at the onset of anaerobiosis, resulting from continued redox cycling (at approximately 7 min), a rapid rate of iron release was observed. Accordingly, lowering the oxygen concentration of the reaction mixture considerably shortened the time required to achieve rapid iron release whereas anaerobiosis at the initiation of the reaction resulted in an immediate release of iron from ferritin. Also, reductive release of ferritin iron by the paraquat radical was much more complete than with O₂⁻ as virtually 100% of the iron could be removed, whereas O₂⁻ appeared to release only approximately 5% of the iron from purified rat liver ferritin (18). The dependency of the time
required for complete release of iron from ferritin on both the activity of NADPH-cytochrome P-450 reductase and the concentration of paraquat provide further evidence that iron release was mediated by the paraquat radical.

The remarkable ability of the paraquat radical to effectively release iron from ferritin suggested that perhaps other bipyr-ridyl radicals would be similarly capable of mobilizing iron from ferritin. Accordingly, our data demonstrate that the cation radicals of benzyl viologen and diquat were also very effective at mediating release of iron from ferritin. It is difficult to assess the effectiveness of the various radicals to release ferritin iron solely on the basis of rates of iron release due to varying rates of reduction of the chemicals by the enzyme; therefore, rates of NADPH oxidation were also monitored. NADPH oxidation rates in the presence of the enzyme and the chemicals are an indicator of the rate of formation of the radical form of the chemicals. The results demonstrated that 2 nmol of NADPH were oxidized per nmol iron released, thus the process was not tightly coupled. This finding is not totally surprising, as iron release from ferritin is highly dependent upon a number of factors including redox potential and size of the reductant (25), and amount of surface area on the iron core available for release (13). Conversely, paraquat can undergo di- or tetravalent reduction or even disproportionation (39), which would significantly affect NADPH oxidation.

The rapidity with which the paraquat cation radical mediates the complete release of iron from ferritin suggests that electron transfer from the radical to the ferric hydroxy ferric core, or to oxidoreduction sites on the protein, occurs very rapidly under anaerobic conditions. Confirmation of this was provided by the ESR and UV-visible spectroscopy studies used to detect paraquat radical formation. The addition of as little as 8 μg/ml ferritin protein (25 μM Fe3+) resulted in a marked diminution of the ESR free radical signal of paraquat, with no detectable signal at 64 μg/ml ferritin. Similar data were obtained when paraquat radical formation was monitored at 603 nm. As the incubations were monitored over time, the paraquat radical could be detected once the iron was released from ferritin. Accordingly, if the ESR reaction mixtures were scanned repetitively over time, a reappearance of the signal was observed as the iron was released from ferritin, thereby allowing an accumulation of the paraquat radical (results not shown). These data indicated that there was significant interaction between the radical form of paraquat and the protein and that loss of the signal was likely due to transfer of electrons from the cation free radical to ferritin.

To provide further evidence that paraquat-dependent release of iron from ferritin occurs by simple electron transfer and does not alter the protein, gel filtration chromatography was used to recover ferritin following its incubation with paraquat and NADPH-cytochrome P-450 reductase under aerobic and anaerobic conditions. Enzyme-linked immunosorbent analysis of fractions collected from the Sephadex G-25 column revealed essentially no change in the reactivity of rabbit anti-ret liver ferritin IgG toward the ferritin (results not shown). In agreement, native and denaturing gel electrophoresis of the ferritin demonstrated there was no effect on the assembled protein or on the individual subunits (data not shown).

It is known that ferritin does have sites sensitive to degradation by proteolysis (44) and pH (45). Preliminary experiments in this laboratory have indicated that iron-free ferritin prepared by incubation of ferritin with the paraquat radical, was much more susceptible to proteolysis. These results agree with other studies (44, 46) which reported that apoferitin is more readily degraded. Therefore, while the paraquat radical may not directly degrade the protein, paraquat intoxication in vivo may lead to disruption of the normal iron storage capacity of cells.

Additionally, iron uptake by ferritin was determined to confirm that the biologic function of ferritin was not compromised by radical-mediated iron release. Iron uptake was greatest for ferritin from which iron was released by the paraquat radical under anaerobic conditions or by dithionite. These results confirm previous work (13) which demonstrated that iron uptake was most rapid for those molecules containing the least iron. The data presented in Fig. 6 have been corrected to account for the iron present in ferritin prior to addition of ferrous iron; thus, while iron uptake was greatest for ferritin containing the least iron, by 6 min all ferritin preparations contained nearly the same amount of iron. Subsequently, the iron in all of the reloaded ferritin preparations could be reductively released by O2 or the paraquat radical (data not shown).

These results provide evidence that the generation of organic free radicals in biological systems can serve to mobilize cellular iron from its storage protein. This finding may help to explain the variable results obtained when using superoxide dismutase to protect against paraquat intoxication (25, 26), i.e. that the relative contributions of O2 and the paraquat radical to paraquat toxicity may be highly dependent upon oxygen concentrations within the cell as has been proposed for carbon tetrachloride (47, 48). It is conceivable that continuous redox cycling of the herbicide may lead to hypoxic conditions which could favor the rapid release of ferritin iron by the paraquat radical while still maintaining a concentration of oxygen sufficient to promote lipid peroxidation. In this regard, it has been reported that hypoxia may potentiate paraquat toxicity in vivo (24). Also, Cadenas et al. (49) observed a burst of chemiluminescence upon oxygensation of microsomes previously incubated anaerobically with NADPH and paraquat. The intensity of chemiluminescence was related to the amount of paraquat radical accumulated during anaerobic incubation.

Barry (50) has reported that human liver iron stores (ferritin) range from 10.8–14.3 nm Fe3+; thus, it is conceivable that interaction between the radical and the protein may occur in vivo. However, the lung is the primary target organ of paraquat toxicity, presumably due to an active uptake mechanism (51). Ferritin is present in almost all tissues at varying levels (62), but the present studies indicate that as little as 8 μg (25 μM Fe3+) of ferritin can readily accept electrons from the paraquat radical; therefore, similar interactions in the lung are feasible. Another factor to be considered with respect to physiologic significance is the subcellular distribution of ferritin. While ferritin is found predominantly in the cytosol, electron microscopy studies have demonstrated the existence of ferritin in the endoplasmic reticulum (53) and, accordingly, ferritin is found in liver and lung2 microsomes prepared by differential centrifugation at 105,000 × g for 90 min. Very recent work indicates that ferritin-bound iron remains tightly associated with the microsomal fraction even following sucrose gradient centrifugation (54). Therefore, it is likely that, in vivo, ferritin is in close proximity to the site of paraquat radical and O2 generation on the endoplasmic reticulum and that little diffusion of these radical species through the cellular milieu to access ferritin is required.

Although the biochemical mechanisms governing the toxicities of paraquat and other redox-active chemicals are likely

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to be very diverse, these data suggest that release of iron from ferritin may be a common feature contributing to free radical-mediated toxicities. It has been recently demonstrated that iron greatly potentiates the toxicity of paraquat in mice (55). Whether this iron is derived ultimately from ferritin remains to be determined, however. These findings would indicate that the administration of appropriate iron chelators may help to ameliorate the toxicity of these compounds. In agreement, EDTA and desferrioxamine prevented paraquat-induced bacterial inactivation (56), and desferrioxamine treatment prolonged survival of paraquat-treated mice (55). These results suggest that redox-active metals such as iron may play an integral role in paraquat toxicity. Thus, the demonstration that the free radicals of paraquat and related toxic herbicides possess the capability to decolorize tissue iron may provide insight into the mechanisms that are involved in the toxicities of these compounds and lead ultimately to more effective prevention and treatment regimens.

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