The Relationship of Intracellular Iron Chelation to the Inhibition
and Regeneration of Human Ribonucleotide Reductase*

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The depletion of cellular iron can lead to the inhibition of ribonucleotide reductase, preventing new DNA synthesis and hence inhibiting cell proliferation. Electron paramagnetic resonance (EPR) spectroscopy has been used to examine simultaneously for the first time the relationship between chelation of intracellular iron and the rate of removal and regeneration of the tyrosyl radical of ribonucleotide reductase within intact human leukemia K562 cells. The different physicochemical characteristics of relatively hydrophobic low molecular weight bidentate hydroxyoxypyrindinone chelators and the higher molecular weight hexadentate ferrioxamine have been exploited to elucidate these interactions further. The base-line concentration of EPR-detectable mononuclear nonheme iron complexes was 3.15 ± 1.05 μM, rising on incubation with chelators more rapidly with hydroxyoxypyrindinones than with desferrioxamine. Hydroxyoxypyrindinones also removed the tyrosyl radical more rapidly, apparently as a consequence of depletion of the intracellular iron pools necessary to regenerate the active enzyme and compatible with their reportedly greater cell toxicity. The radical decay rate is consistent with previous models, suggesting that iron is spontaneously removed from mammalian ribonucleotide reductase. Upon removal of extracellular chelator the regeneration of the tyrosyl radical was significantly faster for hydroxyoxypyrindinones than for desferrioxamine, consistent with their differential effects on cell cycle synchronization.

Ribonucleotide reductase is essential for reduction of ribonucleotides to the deoxyribonucleotides required for DNA synthesis in all organisms (1-3). The enzyme contains two ferric ions (4) coordinated by four carboxylates and two histidine ligands (5), which are antiferromagnetically coupled by a μ-oxo bridge (6, 7) and are therefore EPR-silent. The reaction of the reduced binuclear iron center with oxygen generates a protein-bound tyrosyl radical (8-10). The concentration of this radical correlates with enzyme activity (8). The binuclear iron center also appears to stabilize the radical during steady-state turnover of the enzyme. The iron center and tyrosine radical are buried in the protein (5) but can react with a number of free radical scavengers, antioxidants, and reductants (3, 11, 12). Once formed, the radical in the isolated mammalian enzyme has a half-life (t1/2) of 10 min under anaerobic conditions (12). While mammalian ribonucleotide reductase requires only di-thiothreitol, oxygen, and iron for regeneration of the tyrosyl radical (13, 14), bacterial ribonucleotide reductase appears to require a radical introducing system (11) involving a flavin reductase together with Fe2+ (15) or for iron to be extracted and reintroduced as Fe2+ (6). The mammalian enzyme is found predominantly in the cytosol of cells and has a t1/2 of approximately 3 h; quantities increase 3-7-fold immediately prior to and during S phase of cell cycle as a result of de novo protein synthesis (16, 17).

The Fe3+ chelator desferrioxamine (DFO) is used in the clinical treatment of diseases of iron overload e.g. hemochromatosis, µ-thalassemia (18). It is known that Fe3+ chelators can inhibit the ability of ribonucleotide reductase to reduce ribonucleotides in intact cells at concentrations in the micro-molar range (19, 20). The mechanisms involved are poorly understood, however, as is the exact relationship of ribonucleotide reductase inhibition to the antiproliferative effects of iron chelators. Much of the work concerning the mechanisms of inhibition has been carried out either on isolated bacterial enzyme or on purified, recombinant murine enzyme, with relatively few data on the interactions of chelators within intact cells (21, 22). The kinetics of intracellular ribonucleotide reductase inhibition and regeneration in relation to the formation of intracellular iron-chelate complexes have not been studied.

Given the resistance of the iron center of ribonucleotide reductase to chelation in the E. coli enzyme (23, 24) it seems unlikely that chelators directly remove iron from the binuclear center in the mammalian enzyme. A more plausible explanation for the in vivo inhibitory effects is that intracellular iron chelation deprives the cell of sufficient iron to incorporate into newly synthesized apoenzyme. Recent work has suggested, however, that unlike bacterial ribonucleotide reductase (23), purified recombinant mouse enzyme may lose its iron spontaneously at physiological temperatures (22). These investigators concluded that hexadentate iron chelators, such as desferrioxamine, may act on soluble rather than protein-bound iron by preventing the reincorporation of iron into old, as well as newly synthesized enzyme.

Some iron chelators may inhibit mammalian ribonucleotide reductase, through a mechanism involving redox-active Fe2+...
Conceivable, therefore, that low molecular weight lipophilic direct rather than by long range electron transfer, which tants can gain access to the iron core and alter the redox state previously (27). Purity was confirmed by $^1$H NMR, elemental analysis, methyl-3-hydroxypyridin-4-one (CP20) were synthesized as described 20292 (19). Three molecules of HPO can simultaneously coordinate appear to be more inhibitory to bone marrow from those induced by the hexadentate DFO (19). HPOs also on cell cycle induced by bidentate HPOs differ substantially reductase (19). The mechanism of action is not known and has est (27) that are known to inhibit intracellular ribonucleotide and the iron cannot participate in redox cycling, it is unlikely neutral charge and relative lipophilicity, they may gain access to intracelular iron pools more rapidly than DFO (28).

Low temperature EPR spectroscopy permits the simultaneous detection of both the ribonucleotide reductase tyrosine radical ($g \approx 2.0$) and intracellular iron-chelate complexes ($g \approx 4.3$). Preliminary studies demonstrated that human K562 leukemia cells give a sufficiently strong tyrosine radical signal for the kinetics of inhibition and regeneration to be studied in intact cells (29). We have therefore used this cell line to invesigate the relationship between chelation of intracellular iron and the suppression of the tyrosyl radical of intracellular ribonucleotide reductase. This system will provide information on the mechanisms underlying the apparent differences in toxicity of DFO and the bidentate HPOs.

**MATERIALS AND METHODS**

Reagents—1,2-Diethyl-3-hydroxypyridin-4-one (CP94) and 1,2-di- methyl-3-hydroxypyridin-4-one (CP20) were synthesized as described previously (27). Purity was confirmed by $^1$H NMR, elemental analysis, and reverse phase high pressure liquid chromatography. DFO was purchased from Ciba-Geigy (Basel, Switzerland). $[^{15}$Cl]CP94 (2.405 MBq/mg) and $[^{15}$Cl]DFO (185 Bq/mg) were a kind gift from Dr. H. H. Peter of Ciba-Geigy, RPMI 1640 medium and fetal calf serum were from Life Technologies, Inc. (Paisley, UK) and PBS from Oxoid (Basingstoke, UK). The structure of the chelators used is illustrated in (Fig. 1).

Effect of Iron Chelators on Cell Growth—DFO, CP94, and CP20 were added to cells in exponential growth from a concentrated stock solution in phosphate-buffered saline (PBS). The same volume of PBS was added to control cultures. Since DFO is a hexadentate ligand and coordinates iron in a 1:1 ratio, but the bidentate hydroxypyridin-4-one coordinates in a 3:1 (chelator:iron) manner, activity was compared on a 3:1 molar basis so that equivalent iron binding capacities were achieved, and results are expressed as $\mu$M iron binding equivalents (IBE). 

Cell Culture and Freezing—K562 cells were cultured at 37 °C in RPMI 1640 medium with 5% (v/v) heat-inactivated fetal calf serum under CO$_2$/air (1:19). Cell stocks were maintained at 2-6 × 10$^6$ cells/ml. To ensure that cells were in exponential growth, media were changed 24 h prior to all experiments; cell viability as determined by ethidium bromide/acridine orange fluorescence microscopy was greater than 95% for all experiments. During experiments cells were grown at 4 × 10$^6$/ml (200 ml/flask), and chelator was added from a 33 × concentrated stock.

Cells were frozen in order to study their low temperature EPR spectra. In most studies unstained cells were used for freezing. However, in order to study the effect of the removal of chelator from the incubation medium, at the end of the 24-h incubation period cells were spun down and resuspended in 50 ml of chelator-free medium. At different times, subsequent to this washing, cells were harvested for EPR analysis. Cells were prepared for EPR analysis by centrifuging at 500 × g for 3 min at 37 °C, removing the supernatant, and vortex-mixing the cell pellet. 200-µl aliquots were frozen in EPR tubes. The tubes containing the cells were rapidly frozen (≤ 5 s) in cold methanol and stored in liquid nitrogen. The overall time taken for spinning and freezing was less than 6 min.

Preparation and Analysis of Cell Lysates—K562 cells were spun down into a pellet, and 1-ml quantities of packed cells were placed in microcentrifuge tubes. The cells were probe-sonicated (Soniprep 150, MSE) and treated previously at 30 °C at 5-µm amplitude for 2 × 15 s and put on ice. The cells were microcentrifuged at 13,000 × g for 4 min, and the supernatant was removed. The supernatant was immediately di-vided into three fractions, and CP94, DFO, or control (PBS) was added to the required final chelator concentration of 300 µM IBE. Incubation proceeded at 37 or 4 °C before removal of 200-µl aliquots at the required time points, which were taken into EPR tubes, frozen in liquid nitrogen, and analyzed as described above.

Transferin Labelling—Human apotransferrin was labeled with $^{59}$Fe and saturated to 90% with ferrous ammonium sulfate. $^{59}$Fe and $^{59}$Fe were randomized in a minimal volume of 0.05 m H$_2$SO$_4$ and added dropwise to human apotransferrin dissolved in 1 mM Tris-HCl, pH 8.5, in the presence of 5 mM bicarbonate. After 1 h of incubation at 37 °C, the pink $^{59}$Fe-labeled transferrin solution was dialyzed overnight against 0.05 m Tris-HCl, pH 8.0. The solution was then passed down a 20 × 70-mm diaminoethyl-Sepharose CL6B column and eluted with a linear Tris-HCl/NaCl ionic gradient. The protein-containing fractions were pooled and dialyzed against PBS prior to storage at 4 °C. 

Iron Mobilization from K562 Cells—K562 cells (2 × 10$^4$/ml) were suspended in RPMI 1640 supplemented with 1% bovine serum albumin and incubated with 100 µg/ml $^{59}$Fe-labeled dfferent transferrin at 37 °C for 3 h. After washing with PBS, the $^{59}$Fe-loaded cells were resuspended in culture medium in the presence or absence of 100 µM IBE CP94 or DFO. The cells were incubated at 37 °C and sampled at regular intervals. The samples were centrifuged, and the supernatant was removed and retained. The cells were washed three times with PBS, and the amount of $^{59}$Fe in the cells, supernatant, and each wash was measured using an LKB γ-counter.

Estimation of Trapping of Medium between Cells—For the purposes of calculating intracellular iron-chelate complexes or tyrosyl radical, experiments were performed to determine whether the trapping of
medium between packed K562 cells was appreciable. This would lead to an underestimate of the true intracellular concentrations. Intracellular trapping was measured by mixing 10 μl of 125I (50 μCi/ml) with 1 ml of cells and placing in a Hawksley capillary tube via capillarity, leaving approximately 15 mm unfilled. The dry end was then heat-sealed, and the external surfaces were wiped clean. After centrifugation for 5 min in a Hawksley microhematocrit centrifuge (Gelman Hawksley Limited, Lancing, Sussex, UK), the packed cell volume (PCV) was calculated, the open ends of the tubes were sealed with plasticine, and the tube was cut with a metal file at the interface between cells and the supernatant. The counts in the packed cells and supernatant were determined using a γ-counter. The percentage of trapped plasma (%TP) in the packed cell column (PCC) was calculated with the formula:

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\%TP = \frac{\text{Count rate in PCC} \times (1 - \text{PCV}) \times 100}{\text{Count rate in plasma and PCC} \times \text{PCV}}
\]

(Eq. 1)

The percentage of plasma trapping in these packed cells was 7.8 ± 1.24% from three experiments. It is likely, therefore, that the calculated concentrations of the tyrosyl radical and iron complexes are an underestimate by at most 10%.

EPR Analysis of Cells—Cells were stored in liquid nitrogen and analyzed using a Bruker ESP 300 EPR spectrometer with an Oxford Instruments ESR 900 helium cryostat. Each tube contained 80–100 million cells (0.5 ml total volume). The spectra were recorded at 30 K under nonsaturating conditions: 20 mW for the iron complexes (g = 4.3) and 2 mW for the tyrosyl radical (g = 2). As for the mouse enzyme (13), the electron-spin relaxation properties of the human enzyme were such that it could be readily quantitated at low temperature at this relatively high microwave power. In order to show the structure of the radical, spectra in the g = 2 region were recorded at 0.5-mT modulation amplitude, whereas those at g = 4.3 were recorded with 1-mT modulation amplitude.

The relative concentration of the tyrosine radical under different conditions was obtained by comparing the double integral and peak minus troughs of the signals, measured under nonsaturating conditions. To account for background signals (see Fig. 2), the spectra were always compared with a radical-free spectrum, which was produced by incubation of the chelator of interest with the cells for 24 h at 300 μM 1BE. In order to assess the degree of error in the measurements, four different peak minus trough values were used to analyze the intensity of the signal (332.5–335.7 mT; 332.5–342.7 mT; 338.0–335.7 mT; 331.7–330.7 mT). A complete double integral was also calculated from 329.8 to 338.2 mT. The values from these five measurements were compared, and the means ± S.D. are recorded in the figures. The signal in the absence of chelator addition was arbitrarily set to 1, and the signal following 24-h incubation with 300 μM 1BE of chelators (when no feature of the tyrosyl radical were detectable) was set to 0. Presentation of the results in this manner is designed to illustrate the errors associated with the EPR analysis (especially at lower radical concentrations), but it should be emphasized that the main findings of this paper were readily discernible by simple visual analysis of the EPR spectra alone (see Figs. 2 and 3).

Absolute quantitation of the tyrosine radical was obtained by reference to a Cu-EDTA standard (1.5 mM Cu, 10 mM EDTA). Absolute quantitation of the intracellular high spin ferric chelator complex was obtained by comparing the signal size with a standard curve obtained from the addition of 1–50 μM ferric sulfate to an excess of the chelator. The ferrioxamine signal was sharper than that from Fe3+-CP94 and Fe3+-CP20, but their double integrals were similar if calculated over a wide enough range. They were also equivalent to those seen from the same concentration of ferric transferrin. Therefore, the zero field splitings of these complexes are such that at the temperature of this study (30 K) the states giving the g = 4.3 and the g = 9 signals are virtually equally populated (31). There was no evidence for spin-spin interactions, which can decrease the signal size when metal ions are frozen in ice. Signal-noise ratios were such that concentrations as low as 1 μM iron could be readily detected. The concentration of EPR-detectable iron in the untreated cells reveals a signal with similar hyperfine couplings and microwave power saturation properties to that seen in hydroxyurea-resistant mouse 3T3 cells (13) and human HL 60 cells (32), confirming the ability of the technique to detect human ribonucleotide reductase in vivo. Similar signals were observed in K562 cell extracts; treatment with excess hydroxyurea removed the signal. The ease of detection of ribonucleotide reductase tyrosine radical in the K562 and HL 60 (32) cells suggests that leukemic cell lines overexpress this enzyme. Furthermore, the addition of 2 mM hydroxyurea to the K562 cells for 24 h did not remove the radical signal, demonstrating significant hydroxyurea resistance. The concentration of the radicals in a packed solution of cells (with <10% intercellular volume) was calculated to be 0.22 μM, corresponding to 600,000 molecules/cell. This is significantly higher than that in wild type mouse 3T6 cells and similar to that in TA3H2 mouse cells selected to overproduce ribonucleotide reductase (21). The value is about 15% of the concentration in an overexpressing strain of mouse 3T6 cells (13).

Detection of Intracellular Iron-Chelate Complexes—The same EPR samples used to determine the tyrosine radical concentration can also be used to determine the intracellular ferric-chelator concentration (Fig. 3). The majority of intracellular iron in K562 cells is present in ferritin in which the spin coupling observed between the iron atoms yields a broad EPR signal that is not readily detectable under these conditions (33). However, low molecular weight iron chelates frequently have sharp EPR signals at g = 4.3 (31, 34). In the absence of added chelators a small EPR signal is detected at g = 4.3. The intracellular EPR-detectable iron concentration was calculated to be 3.15 ± 1.05 μM (mean ± S.D., n = 12). Analysis of the growth medium (RPMI 1640 containing 10% fetal calf serum) revealed a similar concentration of EPR-detectable iron. As expected,
the iron in the growth medium had similar spectral features to transferrin, but the intracellular signal was different. This latter signal may be in part due to the cellular low molecular weight iron pool; however, other nonheme iron proteins having $g = 4.3$ signals may also contribute (31).

The addition of DFO or CP94 to cells greatly increases the size of the intracellular $g = 4.3$ signal (Fig. 3). This signal has much greater intensity than the radical and represents chelation of intracellular iron from various sources, not just ribonucleotide reductase. Comparison with the pure chelator-iron complexes revealed that the increase was due to the presence of ferrioxamine and Fe$^{3+}$-CP94 respectively. The cellular signal had identical EPR structure ($g$ value, line shape) to the pure iron-chelate complexes, strongly indicating that the in vivo complexes formed were ferrioxamine and the 3:1 (Fe$^{3+}$:CP94) complex. These were readily quantitated by comparing the cellular signal with those of the pure complexes.

The addition of iron chelators to the cell growth medium, in the presence or absence of cells, did not result in an increase in EPR-detectable iron in the medium, even after 24 h. However, the spectral shape suggested that most of the iron in the case of CP94 (and some of the iron in the case of DFO) was no longer bound to transferrin.

At 30 K, EPR signals were also detected from K562 cells at $g = 1.92$. These did not interfere with the quantitation of either the ferric chelate complexes or the ribonucleotide reductase radical, nor were they affected by iron chelator treatment. They are likely to be from iron-sulfur centers in the mitochondria, probably succinate dehydrogenase and complex I (35).

Kinetics of the Relationship of Tyrosyl Radical Removal to Intracellular Iron-Chelate Formation—Differences were seen between chelators when the time course of enzyme inhibition and intracellular ferric complexes were compared at 100 $\mu$M IBE (Fig. 4). With CP94, the radical was removed rapidly (within 4 h), but a much slower decay was observed with DFO (after 4 h over 50% of the signal remained). The kinetics of the radical decay may have been a consequence of the different rates of chelation of the intracellular iron pools in the two cases. Over 10 $\mu$M iron is bound to CP94 within 10 min of its addition, whereas much slower rates of ferrioxamine accumulation are observed.

Dose Response Relationship of Tyrosyl Radical Inhibition and Iron-Chelate Complex Formation to Concentration of Chelator in Medium—The differences in the ability of CP94 and DFO to remove the ribonucleotide reductase radical essentially disappeared when the compounds were incubated for 24 h with the cells (Fig. 5). Under these conditions both compounds were clearly strong inhibitors of the enzyme. Only a small inhibition with DFO, and none with CP94, was observed at concentrations of 25 $\mu$M IBE or below. At 100 $\mu$M or greater both compounds remove most of the radical signal. At concentrations as high as 300 $\mu$M no radical signals were observed, and thus no ribonucleotide reductase activity (or cell division) was possible.

Differences are observed, however, with the steady-state concentration of intracellular iron chelated over this longer period. Ferrioxamine accumulated inside cells (to between 15 and 20 $\mu$M), whereas the intracellular level of Fe$^{3+}$-CP94 never
rose above 8 μM. Indeed, the intracellular level of Fe^{3+}-CP94 actually slightly decreased from 4- to 24-h incubation (compare Figs. 4 and 5).

Rate of Iron Release with CP94 and DFO Measured by \textsuperscript{59}Fe Labeling—The most likely explanation for the differences in the levels of Fe^{3+}-CP94 and ferrioxamine is that the ferric complex of CP94 is able to leave cells faster than that of desferrioxamine, due to its greater hydrophobicity and lack of positive charge (see Fig. 1). Therefore, Fe^{3+}-CP94 would not build up to the same extent as ferrioxamine. However, it would be more effective at mobilizing iron release from cells, resulting in a drop in intracellular Fe^{3+}-CP94 levels as cellular iron reserves became depleted.

In order to determine if the relative rates of appearance and diminution of the EPR signals for Fe^{3+}-CP94 and DFO reflect the iron-releasing properties of these compounds, K562 cells were pulse-labeled with \textsuperscript{59}Fe transferrin. The relative rates of iron release from cells into the external medium with CP94 or DFO (100 μM IBE) were then compared. Iron release with CP94 was more pronounced and began more rapidly than with DFO (Fig. 6). This rapid early \textsuperscript{59}Fe release into the external medium with CP94 was compatible with the rapid rate at which EPR-detectable CP94-iron complexes formed (Fig. 4). Since chelators have to enter cells first in order to mobilize intracellular iron (36), these differences in iron release are likely to reflect both the relative rates of entry and the rates of egress of the iron-chelate complexes from cells.

Effects of Chelators on Tyrosyl Radical in Cell Extracts—The chelation of the intracellular iron pool has been previously suggested to be responsible for the removal of the RR radical signal (22). The studies above suggest that the increased rate of radical removal with CP94, as opposed to DFO, is primarily due to an increased rate of removal of this readily chelatable iron pool.

To prove this and to study the interaction of iron chelators more directly with the enzyme, we prepared soluble cell extracts from K562 cells, in which the tyrosine radical signal was still readily detectable. In contrast to the purified mouse enzyme (22), the radical signal in the cell extracts was found to be completely stable for 1 h at 4 °C (Fig. 7). Despite the fact that in the absence of a cell membrane CP94 and DFO immediately chelate significant quantities of iron from the cell extracts, they had no effect on the size of the radical signal. Therefore, at 4 °C it seems unlikely that iron is directly removed from the human enzyme, either spontaneously or by chelators. However, at 37 °C both the control and the chelator treated cells showed a decline in the tyrosine radical signal (Fig. 8). After 4 h the radical signal was completely absent in the presence of chelators, whereas the controls still had 30% of their initial level. The time course of this inhibition in cell extracts, both by CP94 and DFO, is similar to that seen in whole cells by the membrane-permeable CP94 and suggests that the removal of intracellular iron pools is the trigger for the removal of the free radical signal rather than direct chelation of the iron from the enzyme. Once the iron pool is chelated, the radical signal decays significantly within 30 min and is completely removed after 2-4 h.
Complex Removal—Incubation with iron chelators like DFO and CP94 blocks K562 cells at the G1-S border of the cell cycle (19). Removal of the chelators from the cell culture medium causes an increase in the proportion of cells in S-phase within 4–8 h as cells return to cell cycle in a synchronized manner (19). This effect is more marked with CP94 than with DFO and it was postulated that this is due to the more rapid removal of CP94 and its ferric complex from cells (19). As ribonucleotide reductase production is increased in S-phase, one might predict an increase in the ribonucleotide reductase radical signal during the removal of chelator from K562 cells over and above that seen in control untreated cells.

Fig. 9 shows such “offset” experiments. The intracellular concentration of ferric CP94 dropped rapidly (within 10 min). The removal of the chelator, which is known to trigger a significant increase in cells in the S-phase (19), increased the ribonucleotide reductase radical signal over the first 3 h, reaching a peak almost 4 times that seen in untreated unsynchronized cells. By 24 h, the cells had returned to normal, unsynchronized control levels. Mouse TA 3 cells have been found to show a 3–7-fold increase in ribonucleotide reductase content upon entering S-phase from G1 (17). Compared with untreated K562 cells, the number of cells in S-phase approximately doubles upon removal of CP94 (19). Therefore, the increase in ribonucleotide reductase seen when cells go from G1 to S is very similar for mouse and human cell lines.

The efflux of ferrioxamine was much slower than that of Fe3+-CP94, since no significant drop in the ferrioxamine EPR
signal was seen in the first 3 h. Consequently, a slower rise was observed in the radical signal, although it still rose significantly above that observed in control cells by 10 h. By 24 h the radical signal had returned to control levels, although there was still detectable intracellular ferrioxamine. The fact that significant radical signal was observed after 3 h, when there was no detectable drop in intracellular ferrioxamine, suggests that the efflux of desferrioxamine is more rapid than that of ferrioxamine.

Comparison of CP94 and CP20 on the Tyrosine Radical and Intracellular Iron Chelation—CP94 and CP20 have undergone clinical trials as orally active iron chelators to replace DFO in the treatment of hemochromatosis and β-thalassemia (18). Due to their different structures and partition coefficients, one might expect them to have different rates of radical removal and intracellular ferrioxamine. The fact that significant radical signal was observed after 3 h, when there was no detectable drop in intracellular ferrioxamine, suggests that the efflux of desferrioxamine is more rapid than that of ferrioxamine.

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At 300 μM IBE both hydroxypyridinones show complete removal of the tyrosine radical after 4-h treatment (Fig. 10). The kinetics of this inhibition are similar in the two cases; presumably the similar partition coefficients (Fig. 1) lead to similar uptake kinetics of the unchelated forms of CP20 and CP94. There are clear differences in the partition coefficients of the chelated complexes, with Fe₃⁺-CP20 being much more hydrophilic than Fe₃⁺-CP94. This is reflected in the much greater accumulation of the Fe₃⁺-CP20 within cells. After 4 h the Fe₃⁺-CP20 complex accumulated to 4 times the concentration of Fe₃⁺-CP94. After 24 h the [Fe₃⁺-CP20] was over 70 μM, significantly higher than even that of the charged ferrioxamine (Fig. 5).

The suggestion that Fe₃⁺-CP20 cannot readily leave cells is further demonstrated by the kinetics of radical regeneration and iron removal, once the free chelates are removed from the extracellular medium (Fig. 11). While the intracellular [Fe₃⁺-CP94] was undetectable after 10 min, the [Fe₃⁺-CP20] remained elevated for over 1 h. However, the slowness of the removal of the chelated complex did not affect the regeneration of the ribonucleotide reductase radical. In fact this was even
quicker in the case of CP20 removal than CP94 removal. This demonstrates clearly that the unchelated CP20 is rapidly membrane-permeable, but the chelated complex is not. Furthermore the chelated complex is unable to significantly inhibit ribonucleotide reductase; despite the high concentration and slow removal of the chelated complex, physiological low molecular weight iron pools can reform rapidly once the unchelated complex is removed. This requires the \([\text{Fe}^{3+}\cdot\text{CP20}]\) complex to possess a high stability in cells, which is likely in the pH range 6.5–7.5.

The kinetics of reformation of the ribonucleotide reductase radical signal provides further evidence to support the contention that there is no direct interaction of the iron chelators with the enzyme (22). Fig. 11 shows that the unchelated CP20 can cross cell membranes on a time scale of minutes; yet, following chelator addition, full inhibition takes 2–4 h (Fig. 10). The removal of the radical is therefore an event that occurs at a defined rate, independent of the presence of chelators, but in their presence radical regeneration is prevented. Either iron is spontaneously being removed from the enzyme at a slow rate as suggested by Nyholm et al. (22), or the polypeptide chain is itself turning over.

**DISCUSSION**

The presence of a stable free radical in active ribonucleotide reductase, and the fact that most low molecular weight ferric iron chelators form high spin complexes, make EPR an invaluable tool for studying the interaction of iron chelators with ribonucleotide reductase and intracellular iron pools. An understanding of these processes is clinically relevant, because differences in the rate of RR inhibition between DFOs and HPOs could account for the unwanted effects on white cells seen with CP20 in clinical trials (18).

For the chelator in current clinical use, DFO, the kinetics for suppression of the tyrosyl radical appear consistent with the rate that would be predicted if incorporation of iron into newly synthesized protein were inhibited. The half-life of the R2 protein was estimated to be 3 h (16), and this is similar to the rate of decline of the radical seen in K562 cells incubated with DFO (Fig. 4). This is also similar to that observed in hydroxyurea-resistant murine TA3 cells treated with DFO (22).

However, an explanation based solely on turnover of the R2 protein appears inconsistent with the data observed from the small hydrophobic chelators, CP20 and CP94. These compounds remove the radical more quickly than DFO. We attribute this primarily to the increased membrane permeability of their unchelated form, since the studies on soluble cell extracts suggest that there is no difference in the radical removal rate between iron chelators once the membrane has been removed. Our simultaneous, direct observation of intracellular iron complexes and the tyrosine radical in cells shows that whenever ribonucleotide reductase is inhibited, intracellular iron-chelates are detected. However, significant inhibition is only observed when relatively high concentrations (\(\geq 5 \mu M\)) of iron complex are present, suggesting that the removal of the soluble iron pools is a prerequisite for the inhibition of RR.

Once a significant fraction of the low molecular weight iron pool has been removed it appears that a set of events is put into place that reduces the steady-state radical concentration; this process occurs at an essentially chelator-independent rate. Experiments using a high concentration of the most permeable chelator (CP20) suggest a \(t_{1/2}\) for this process of about 30 min inside cells (Fig. 10). This is consistent with the \(t_{1/2}\) for radical removal in the cell extracts (Fig. 8). More importantly, this rate is similar to that found for iron removal from recombinant murine RR, trace-labeled in vitro with \(^{59}\text{Fe}\) (22). It appears to be too fast to be due to turnover of the polypeptide subunits themselves. We therefore agree with the (unexpected) suggestion from Nyholm et al. (22) that mammalian RR spontaneously loses its iron center at physiological temperatures. The iron removal is likely to have a specific mechanism, given the apparent inability of small hydrophobic chelators, such as the hydroxypyridinones, to chelate iron directly from the enzyme.

An independent demonstration of the liability of the iron in this enzyme is highly significant, given previous controversies over pulse labeling. For example, in vitro labeling of other proteins (such as transferrin) has shown that it is possible that some \(^{59}\text{Fe}\) can be nonspecifically bound during the labeling process and that subsequent loss is not from a physiologically relevant site.

The IC\(_{50}\) of the tyrosyl radical as estimated by EPR spectroscopy at 24 h in intact K562 cells is approximately 90 \(\mu M\) IBE for both DFO and CP94. This is an order of magnitude greater than the steady-state plasma concentration of DFO (7 \(\mu M\)) achieved clinically after infusion at conventional doses of 50 mg/kg in iron-overloaded patients (37). In contrast, the concentrations of the HPOs CP20 and CP94 are considerably in excess of this, reaching up to 250 \(\mu M\) (72 \(\mu M\) IBE) following single oral doses of CP20 at 50 mg/kg (38) and approximately 166 \(\mu M\) (56 \(\mu M\) IBE) with CP94 (39). We have detected relatively high concentrations of the intracellular tyrosyl radical in K562 cells; it is likely that concentration in other cells is less than that in K562 cells, thereby increasing their sensitivity to chelation of the low molecular weight iron pool. For example, previous studies have shown that Daudi cells as well as murine erythroid and myeloid colonies have a lower IC\(_{50}\) for cell proliferation than K562 cells (19, 28, 40). It is also possible, however, that the antiproliferative actions of iron chelators are not entirely secondary to inhibition of RR. The IC\(_{50}\) for K562 cell proliferation, DNA synthesis, and CDP reduction by CP94 was approximately 20 \(\mu M\) IBE (19, 39), whereas the IC\(_{50}\) for the RR tyrosyl radical is approximately 90 \(\mu M\) IBE. One explanation for this difference may be that only a relatively modest decrease in the tyrosyl radical results in a proportionately greater inhibition of ribonucleotide reduction in vivo.

The IC\(_{50}\) for the RR tyrosyl radical inhibition by DFO or CP94 is significantly greater than the likely \((<5 \mu M)\) steady-state levels of the low molecular weight iron pool in the cell. It is possible that chelation of this iron pool is not in itself sufficient for RR inhibition; for instance, a component of the intracellular iron necessary for generation and maintenance of the RR tyrosyl radical could be relatively inaccessible to these compounds at low concentrations. However, the low molecular weight iron pool is not a static entity, and it seems more likely that the rate of generation of the low molecular weight iron pool may be such that a relatively high concentration of chelator is necessary to ensure that the steady-state concentration of the pool is reduced to levels that inhibit RR activity.

The contrasting patterns of regeneration of the tyrosyl radical and of removal of iron-chelator complexes after treatment with DFO compared with CP94 shed some light on previous observations. The finding that the tyrosyl radical signal reaches concentrations 3–4 times in excess of control cells between 2 and 4 h after removing CP94 from the medium (and 10 h after with DFO) (Fig. 9) is compatible with the effects of these compounds on cell cycle synchronization (19), since it is known that the tyrosyl radical increases 3–7-fold during G1/S phase (17). We have previously shown that a 24-h incubation of K562 cells with HPOs or DFO arrests cell cycle at the G1/S border and that, after washing, CP94-treated cells return to cell cycle in a synchronized manner, whereas DFO-treated cells return to cell cycle with a less pronounced synchronization (19). The rapid return of the tyrosyl radical within 5 min of removal
of CP94 and CP20, contrasting with the 2 h taken in DFO-treated cells, is compatible with the hypothesis that the uncharged lipophilic hydroxy pyridinones are able to diffuse out of cells more rapidly than the positively charged hydrophilic DFO, thereby allowing a more pronounced cell cycle synchronization (28).

The EPR measurements of the iron-chelate complexes in this study also support previous observations where, using 14C-labeled chelators and 59Fe labeled cells, we found that iron complexes of DFO, unlike those of CP94, accumulate within the cytosol of K562 cells after several hours (28). In the present study, the EPR signal of the CP94-iron complex fell within 5 min of cell washing, in contrast to the ferrioxamine signal and the CP20-iron complex, which were detectable for much longer (Figs. 9 and 11). Secondly, the steady-state concentration of CP20-iron was 3 times greater than that of ferrioxamine after 24 h of continuous incubation; ferrioxamine accumulation was in turn 3 times that of the CP94-iron complexes. These differences may be explained by the relative physicochemical properties of the Fe3+ chelate complexes. The unipositive charge of DFO is unaltered by binding Fe3+, as is the neutral charge of HPOs (36). Furthermore, ferrioxamine, and especially Fe3+ -CP20, remain relatively hydrophilic compared with CP94-iron complexes (27, 36). Hydrophobic and/or uncharged iron chelators, such as the hydroxy pyridinones, will rapidly gain access to the cell; however, effective iron mobilization also requires that the iron-chelate complex be uncharged and hydrophobic. This is true of CP94 but not of CP20, with the consequence that the Fe3+ -CP20 complex accumulates intracellularly.

The consequence of the mechanism of RR radical removal determined in this study is that any iron chelator will inhibit ribonucleotide reductase activity to some extent. An ideal chelator for clinical use will be hydrophobic and neutral in both the chelated and unchelated forms and thus able to mobilize iron from cells quickly enough to be usable at chelator concentrations at which ribonucleotide reductase is only weakly inhibited.

We have shown that EPR spectroscopy can yield useful information about the rate of formation and efflux of exogenous low molecular weight iron complexes in cells; intriguingly, we have also detected low concentrations (3 mM) of endogenous mononuclear nonheme iron complexes. Further studies will reveal whether the technique can shed any light on the nature of the endogenous intracellular low molecular weight iron pool.

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