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The Iron-selective Chelator Desferal Can Reduce Chelated Copper

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It was shown that the iron-selective chelator desferal (desferrioxamine mesylate: DFO) can reduce Cu(II) as judged by measuring the formation of the complex between Cu(I) and a specific chelator for this species, neocuproine (NC), in phosphate buffer. It was found that under optimal conditions, 3 moles of Cu(II) could be reduced per mole of DFO. Studies of the kinetics of Cu(II) reduction by DFO revealed that the rate of Cu(II) reduction by DFO was considerably slower than that by ascorbate. In the case of both reductants, even in the absence of NC, Cu(I) complexes remained in aqueous solutions for at least 30 min. DFO could also reduce Cu complexed to histidine. The results presented highlight the interpretive dangers which can arise in studies involving multiple transition metals, especially in the presence of multiple chelators. Specifically, when desferal is used, it is important to be aware that any copper present may become reduced, and that any Cu(I) formed might participate in ongoing redox reactions.

Key words: desferrioxamine, copper, metal chelator, transition metals, redox reactions, neocuproine.

INTRODUCTION

The iron-selective chelator desferal (desferrioxamine mesylate; Ciba-Geigy: DFO) is widely used to prevent iron-dependent free radical reactions.[1] It is sometimes considered that desferal solely interferes with reactions of iron, amongst transition metals.[1] In fact it can also efficiently prevent copper-dependent reactions[2,3] (though this is understated in the abstracts of these papers). The stability constants of desferal for Fe(III) and Cu(II) are respectively 10^{31} and 10^{14}.[4]

It has been appreciated for some time that both iron and copper are probably important in free radical reactions *in vivo*, especially when they become displaced from their normal binding sites on specialised proteins, such as transferrin and caeruloplasmin.[5] Pro-oxidative interactions between these two metals have also been described.[6,7] Thus, experimental situations increasingly arise in which one wishes to study the actions of iron in the presence of copper. This also arises inevitably when one studies tissue, or minerals such as asbestos, which contain both metals. Thus desferal is often used when both iron and copper are present.

We show here that desferal itself can cause the formation of reduced copper complexes

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detectable with the selective Cu(I) chelator, neocuproine. This capacity is long-lived even in the absence of neocuproine, in spite of the well known instability of Cu(I) in aqueous solution. This observation may have practical importance because in many circumstances the supply of reduced transition metal seems to be a critical determinant of ongoing pro-oxidative events. For example, in transition metal-peroxide radical generating systems, providing a metal reductant can often enhance the rate of radical generation, which is otherwise restricted by the limited supply of reduced metal.[8]

MATERIAL AND METHODS

$CuCl_2 \cdot 2H_2O$ was purchased from BDH, desferrioxamine mesylate (Desferal, DFO, additive-free) from Ciba-Geigy, and neocuproine hydrochloride (NC), bathocuproinedisulfonic acid (as the sodium salt), DL-histidine and L-ascorbic acid were purchased from Sigma.

To measure the stoichiometry of formation of reduced copper due to the presence of DFO, 2 mL samples of 100 μM $CuCl_2 \cdot 2H_2O$, in the presence of 250 μM NC, were prepared in 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4). DFO (0–200 μM) was added and the samples incubated at room temperature. At 60 and 120 min after addition of DFO, formation of the Cu(I)/NC complex was assayed by measuring the absorbance at 454 nm using a Hitachi U-1100 spectrophotometer. Samples were blanked against NC alone in phosphate buffer (blank absorbances were less than 0.003). Absorbance readings for Cu(I)/NC complexes were converted into Cu(I) concentrations by means of the published extinction coefficient ($7.95 \times 10^3\ M^{-1}\ cm^{-1}$),[9] and this value was confirmed by means of standard curves for formation of Cu(I) by ascorbate.

For the time course of DFO-mediated reduction of Cu(II), 2 mL samples of 100 μM $CuCl_2 \cdot 2H_2O$ in phosphate buffer were assayed. The reaction was initiated by the addition of either 25 μM DFO or 50 μM ascorbate. 250 μM NC was present at the time of addition of the reductant or added 30 min later. In some experiments, histidine was present prior to the addition of reductant. The increase in absorbance at 454 nm was measured at 4 min intervals using a Beckman DU-65 spectrophotometer with a Kinetics Soft-Pac Module (Beckman). As with the stoichiometry results, the absorbance readings were converted into Cu(I) concentrations using the published extinction coefficient. Samples were blanked against NC alone in phosphate buffer.

RESULTS AND DISCUSSION

Figure 1 shows that in phosphate buffer solution, Cu(II) alone gives rise to virtually no Cu(I)/NC complexes (detected by absorbance). However, when desferal is added to the system, a dose dependent formation of such Cu(I) complexes is observed. The characteristic absorbance at 454 nm is not formed when DFO and NC are added to buffer without copper. Further, the spectra of the Cu(I)/NC complexes formed in the presence of desferal are indistinguishable from those formed by Cu(II) and NC in the presence of reductants such as ascorbate, which are used to standardise the Cu(I)/NC complexes. No similar absorbing species is formed by the combination of Cu(II) and DFO nor if Cu(II) is reduced by ascorbate in the presence of DFO alone (data not shown). Thus the absorbing species measured in Figure 1 is clearly the Cu(I)/NC complex.

The stoichiometry of the formation of Cu(I)/NC with varying concentrations of DFO was complex. At 25 μM DFO, maximal reduction of the available copper was found and corresponded to the formation of approximately 75 μM Cu(I), suggesting that one DFO molecule can reduce at the most three copper atoms, consistent with the presence of three hydroxamate functions on the chelator. The suprising decline in the formation of Cu(I)/NC complex at higher DFO concentrations may be due to a variety of complex

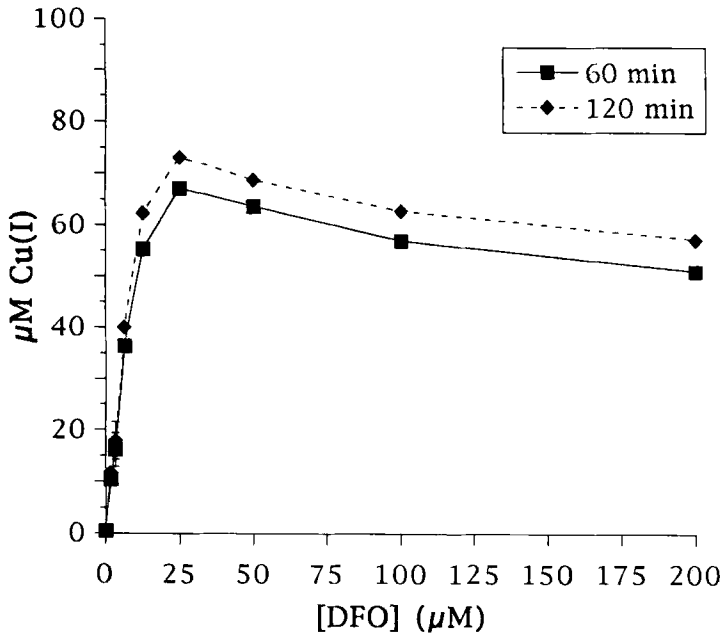

FIGURE 1 Concentration dependence of reduction of Cu(II) by desferrioxamine (DFO). 100 μM $CuCl_2 \cdot 2H_2O$ was incubated in 10 mM potassium phosphate buffer (pH 7.4) in the presence of 250 μM neocuproine and various concentrations of DFO for 60 and 120 min at room temperature before absorption at 454 nm was measured. Result are expressed as the mean of 2–5 (60 min) and 2–3 (120 min) independent experiments, each involving triplicates, ± standard error of the mean.

equilibria between the different chelated states of the copper present in the system. For example, Cu(I) may itself remain associated with the DFO when DFO is in excess, and thus not be observed as Cu(I)/NC complexes. This points to the question whether DFO reduces copper supplied without any other chelator, or only copper which is already chelated (as the Cu(II) species) to neocuproine.

Figure 2 indicates that even when NC was added to DFO/Cu(II) mixtures only after a delay of 30 min, substantial formation of Cu(I) complexes occurs, and eventually closely approximates the absorbances observed in the incubations which were complete at the outset. This suggests that similar equilibria are involved in both cases, and that significant Cu(I)/DFO complex exists in the Cu(II) and DFO solution without NC at 30 min. Thus, in the presence of DFO, the pool of Cu(I) formed in phosphate buffer seems to be maintained for at least 30 minutes. In other experiments, not shown, we have observed its maintenance for more than 60 min. Qualitatively similar results have been observed in experiments with the alternative Cu(I) chelator, bathocuproine sulfonate, which forms a complex with peak absorption at 482 nm.

It remains possible that under the conditions of Figure 2, copper reduction is only initiated when NC is added. However, the additional observations in Figure 2 concerning copper reduction in the presence of ascorbate, confirm that Cu(I) can be formed prior to the addition of NC, and yet remain available to it; we suspect that this correctly describes the situation with DFO and Cu(II). Figure 2 shows that ascorbate reduces virtually all of the available copper very rapidly. Separate experiments indicate that the reduction occurs

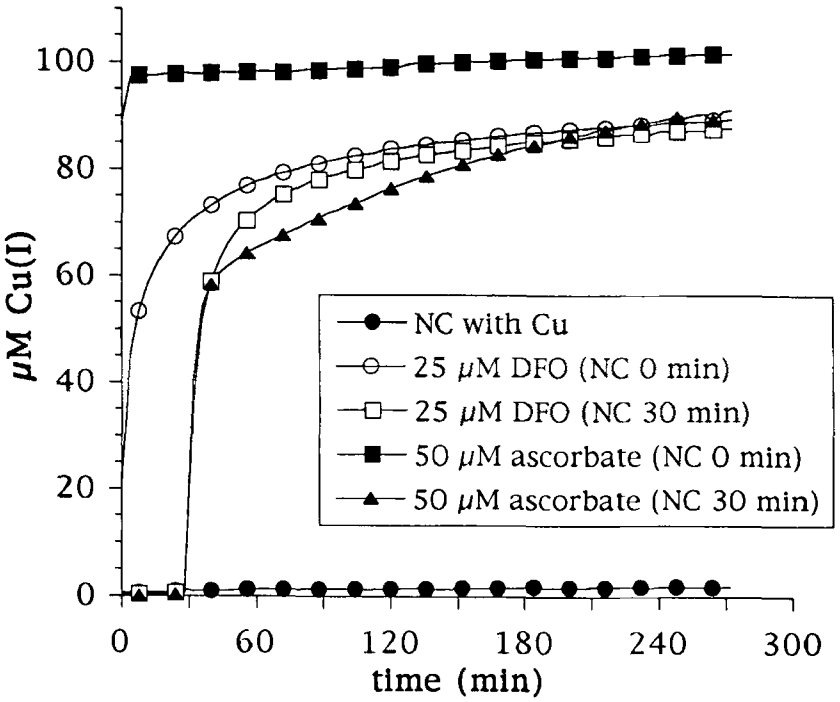

FIGURE 2 Reduction of Cu(II) over time by desferrioxamine or ascorbate. 100 μM $CuCl_2 \cdot 2H_2O$ incubated in 10 mM potassium phosphate buffer (pH 7.4) and was treated with either desferrioxamine (DFO, 25 μM) or ascorbate (50 μM). 250 μM neocuproine (NC) was present from the start of the incubation ('0 min') or added 30 min after addition of the reductant ('30 min'). Results are from one of three representative experiments and readings are corrected against the NC blank at each time point. The graph records the increase in Cu(I) concentration at 4 min intervals, but for clarity only selected points are indicated.

within 1 min in such a system. Similarly, the addition of NC after 30 min of incubation of Cu(II) and ascorbate (solid triangles in Figure 2), causes relatively rapid formation of Cu(I)/NC complexes, rather similar in pace to that observed in the comparable condition with DFO as reductant. Even after 4 hr, the Cu(I)/NC absorbance is still increasing in the ascorbate incubation to which NC was only added at 30 min, and is doing so more rapidly than the Cu(I)/NC absorbance is increasing in the control lacking reductant.

The simplest explanation of these results with ascorbate is that in the absence of NC, Cu(I)/ascorbate complexes of some stability are formed during the first 30 min, and the reduced copper is subsequently slowly transferred to NC. These observations are consistent with the classic studies of Martell and others[10] revealing the complexities of metal-catalysed ascorbate oxidation, which can clearly occur in the absence of an additional Cu(I) chelator.[3] Furthermore, some of the products of ascorbate oxidation may themselves be reductants, and responsible for continued formation of reduced copper available to form NC complexes. We have also noted (unpublished data) that copper reduced by protein-bound DOPA (3,4-dihydroxy-phenylalanine, a product of radical attack on proteins[11,12]) is at least as successfully trapped by NC after a 30 minute delay as when NC is present throughout.

The reduction by DFO in our complete system (NC present from the outset), is slower than that due to ascorbate, and forms around 75 μM Cu(I)/NC after 60 min, and continues slowly

thereafter. The subsequent reaction may be primarily the continuing displacement of Cu(I) from complexes with DFO onto NC. There is a very slight progressive formation of Cu(I)/NC in the control without reductant (Figure 2), and such a phenomenon is well known[13] and probably due to the thermodynamic favouring of the Cu(I)/NC complex over other metal complexes (reduced or oxidised) in the system.

It is important to consider whether the reduction of Cu(II) by DFO can take place when the copper is chelated by relevant biomolecules. Figure 3 indeed shows that when Cu is chelated with various concentrations of histidine, one of its most important biological chelators, reduction by DFO (added subsequently) can be observed. In the first 30 minutes of relatively rapid reduction, the extent of formation of Cu(I)/NC was inversely dependent on the histidine concentration. Similarly, the slower reduction rates observed thereafter were also inversely related to the histidine concentration, and this was confirmed in a separate experiment (not shown), in which histidine concentrations up to 1 mM were included. Thus copper reduction by DFO can occur in the presence of at least some biological chelators.

Joshi and colleagues[14,15] have also demonstrated redox-activity of copper/DFO complexes, in terms of DNA cleavage. However, they found an absolute requirement for the presence of reductant (dithiothreitol, mercaptoethanol or ascorbate) in the system: Cu/DFO alone did not cleave DNA. In view of our data, one would expect Cu(I) to be present even without reductant, but also, that the availability of appropriate Cu(I) chelating sites separate from the DFO might be needed to permit the Cu(I) to act on the DNA. The latter can be provided by the reductant, in view of our observations above on ascorbate. Without such a chelating site, Cu(I) presumably remains associated with the DFO.

Substantial ferric iron reduction by aged or heat treated DFO solutions has also been described recently.[16] The iron reduction observed with native DFO in that study was less than 0.2

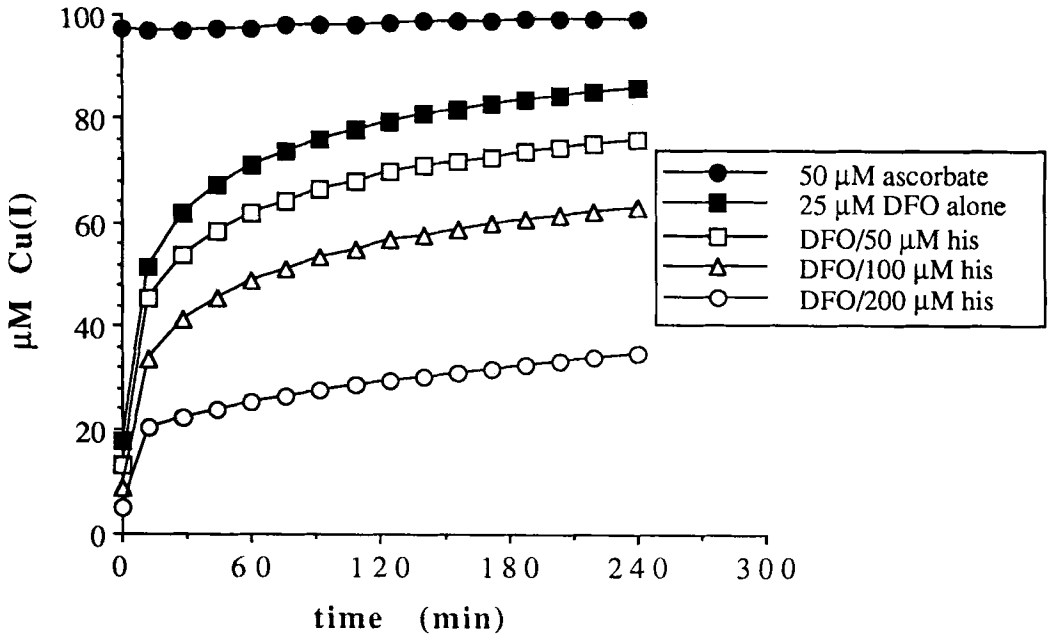

FIGURE 3 Reduction of Cu(II)/histidine complexes. The experiment was conducted as for Figure 2, except that where shown, histidine at 50 µM, 100 µM or 200 µM was present prior to the addition of desferal. Results are from a single representative experiment and are presented as for Figure 2.

mole equivalents per mole of DFO, whereas we observed 3 mole equivalents of reduced copper. In addition, we have only worked with freshly dissolved DFO, obtained directly from the manufacturer, and have made similar observations with two different batches. Thus our observations cannot be explained by the generation of damaged DFO molecules, unlike the data of Gutteridge and colleagues.[16]

Clearly these apparently simple systems are actually very complex in terms of the redox state of the metal contained, and the equilibria between the several chelated forms of the metal, in both its reduced and oxidised forms. We have not attempted to elucidate these systems further, but rather wish to point out the interpretive dangers which can arise when multiple transition metals are studied, especially in the presence of multiple chelators. Specifically, when the common iron chelator, desferal, is used, it is important to be aware that any copper present may become reduced, since such Cu(I) may be important for ongoing redox reactions.

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