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QUANTITATIVE EFFECTS OF IRON CHELATORS ON HYDROXYL RADICAL PRODUCTION BY THE SUPEROXIDE-DRIVEN FENTON REACTION

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ABSTRACT

Iron bound to certain chelators is known to promote the conversion of superoxide radicals (O_2^-) to hydroxyl radicals (HO') by the superoxide-driven Fenton reaction. The production of HO' by various iron chelates was studied using the reaction of dimethyl sulfoxide and HO' to produce methane sulphinic acid. Methane sulphinic acid was quantified by use of a simple colorimetric assay and used to determine the amounts of HO' produced. Superoxide was generated from 200 µM hypoxanthine and 0.05 U/ml xanthine oxidase in the presence of 0-100 µM iron and 100 µM of each chelator. The results of this preliminary investigation illustrate that, at physiological pH, the superoxide-driven Fenton reaction is significantly promoted by iron chelated to EDTA, nitrilotriacetate, and citrate, but is not promoted by the other anions studied.

KEY WORDS: Citrate, deferoxamine, EDTA, nitrilotriacetate, superoxide, xanthine oxidase.

NOMENCLATURE: ADP: adenosine diphosphate; DMSO: dimethyl sulphoxide; EDTA: ethylenediaminete-traacetic acid: NTA: nitrilotriacetate; MSA: methane sulphinic acid.

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INTRODUCTION

A currently popular hypothesis explaining the toxicity of superoxide radicals (O_2^-) in biological systems is is that the O_2^- is converted in the presence of iron to highly toxic hydroxyl radicals (HO') via the superoxide driven Fenton reaction:

$$
\begin{aligned}
O_2^- + Fe^{+++} &\rightarrow O_2 + Fe^{++} \\
2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2 \\
H^+ + Fe^{++} + H_2O_2 &\rightarrow HO^{\cdot} + Fe^{+++} + H_2O.
\end{aligned}
$$

Ferric iron is sparingly soluble at a pH of 7.4 (K_{sp} for $Fe(OH)_3 = 1 \times 10^{-36}$) [6]. Therefore, for the above reactions to be biologically significant, the Fe^{+3} must be chelated to remain in solution. Perhaps, when referring to biological systems it is more appropriate to write the above reactions as:

$$
\begin{aligned}
O_2^- + A\text{–}Fe^{3-n} &\rightarrow O_2 + A\text{–}Fe^{2-n} \\
2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2 \\
H^+ + A\text{–}Fe^{2-n} + H_2O_2 &\rightarrow HO^{\cdot} + A\text{–}Fe^{3-n} + H_2O,
\end{aligned}
$$

in which iron is shown complexed to a chelator anion A^{-n}. The development of a simple, inexpensive method of measuring HO' in our laboratory[7] facilitated a re-examination of the effects of various iron chelators on Fenton chemistry. In this method the reaction of HO' radicals with dimethyl sulphoxide (DMSO) to form methane sulphnic acid (MSA) [8-11] is used to determine HO' quantitatively: $CH_3\text{-}SO\text{-}CH_3 + HO' \rightarrow CH_3SOOH + CH'_3$).

Dimethyl sulphoxide concentrations of 0.1 to 1.0 M can be used to ensure efficient trapping of nearly 100 percent of HO' radicals generated [12-14] without inhibition of enzymes, such as xanthine oxidase, that generate superoxide. Methane sulphinic acid is detected using a diazonium salt, Fast Blue BB, which forms a yellow complex with the sulphinate anion. This complex can be extracted into an organic solvent and detected spectrophotometrically. The objective of the present investigation was to study the role of iron in the presence of various chelators on the production of hydroxyl radicals from superoxide.

MATERIALS AND METHODS

Materials were purchased from the following sources: ADP, albumin, hypoxanthine, phytic acid, uric acid, and xanthine oxidase from Sigma Chemical Co. (St. Louis, MO), nitrilotriacetic acid and Fast Blue BB dye from Aldrich Chemicals (Milwaukee, WI), methane sulphinic acid from Fairfield Chemical Company (Blythewood, SC), deferoxamine from Ciba-Geigy, (Summit, NJ), DMSO, EDTA, sodium citrate, Na_2HPO_4 and NaH_2PO_4 from Fisher (Itasca, IL), and $FeC1_3$ from Mallinckrodt (Paris, KY). Absorbances were measured using a Perkin Elmer Lamba 3B spectrophotometer (Norwald, CN).

Superoxide was generated from hypoxanthine and xanthine oxidase in 0.04 M sodium phosphate buffer (pH 7.4), in the presence of 1.0 M DMSO. Stock $FeC1_3$ solutions were prepared in pH 2 HCl; stock solutions of the chelators in the buffer. All solutions were prepared using 5 to 18 MΩ water, pH 7.0, obtained from a Culligan D-45-P reverse osmosis system. Final concentrations in the 2 ml reaction volume were 100 μM chelator (albumin was 0.4 g/ml), 0.2 mM hypoxanthine, 0.05 U/ml xanthine oxidase, and 1.0 M DMSO. Fe^{+3} concentrations varied from 0-100 μM. In addition to the chelator anions being studied, all experimental solutions included 4×10^{-2} M phosphate (present as Na_2HPO_4 and NaH_2PO_4) as well as chloride varying from 1.3×10^{-4} to 7.0×10^{-4} M (since the Fe^{+3} was added as a solution of $FeC1_3$ in HCl).

Tubes were prepared by adding, in order, the buffer, DMSO, chelator, $FeC1_3$, and hypoxanthine. The reaction was initiated by adding the xanthine oxidase, mixed for 1 min, and incubated at room temperature for 20 minutes. The assay for methane sulphinic acid was a modification of a previously published method [7]. The pH of the sample was lowered to 2.5, 100 μL of 30 mM Fast Blue BB dye was added and the solution incubated at room temperature for 10 minutes. The methane sulphinic acid-dye complex was then extracted into 1 ml of a 3:1 mixture of toluene and butanol. The toluene:butanol mixture was washed with 2 ml of butanol-saturated water. One hundred microliters of 5% glacial acetic acid in pyridine was added to the organic phase to stabilize the color, and the absorbance of the organic phase at 420 nm determined.

RESULTS

The effects of added iron in the presence of various chelator anions upon HO' generation by the superoxide-driven Fenton reaction are shown in Figure 1. Iron added to solutions of phytate, ADP, urate, and albumin catalyzed only minimal formation of methane sulphinic acid, which was not significantly different from that produced in the presence of phosphate buffer alone. However, methane sulphinic acid generation in the presence of EDTA, NTA, and citrate anions was substantially higher than that in the phosphate buffer alone for all concentrations of iron. Even at zero added iron, the addition of EDTA caused production of methane sulphinic acid, perhaps by chelating trace amounts of Fe^{+3} in the reagents. Methane sulphinic acid production was not detected in the presence of 100 μM deferoxamine. Samples containing all the reagents except xanthine oxidase did not produce detectable amount of methane sulphinic acid (results not shown), verifying that the response required the presence of enzymatically generated O_2^-.

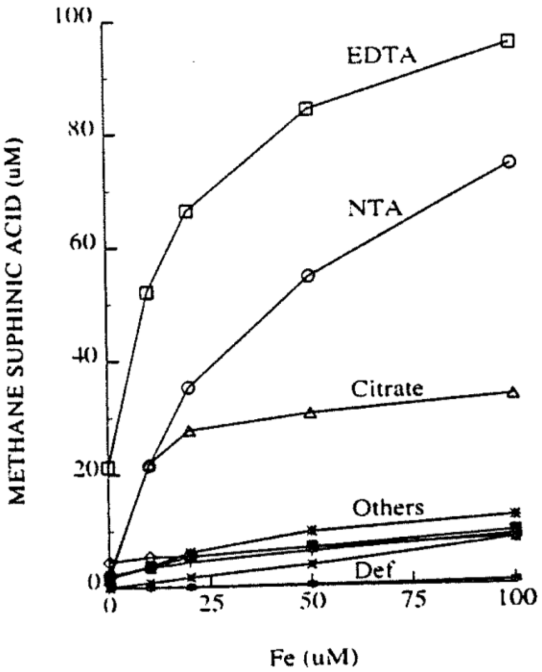

FIGURE 1 Hydroxyl radical production from O_2^- produced from the reaction of xanthine oxidase and hypoxanthine, with varying concentrations of iron, measured as MSA production in 1 M dimethyl sulphoxide, in the presence of 100 μM of various chelators. Data points represent the means of triplicate determinations. Standard deviations for all the experiments were less than 5 μM methane sulphinic acid,except when Fe = 100 μM in the presence of EDTA (SD = 11 μM). Included in "others" are phosphate buffer alone, albumin, ADP, urate, and phytate. NTA = nitrilotriacetate, Def = deferoxamine.

DISCUSSION

In addition to previous reports [7, 14, 15], the experiments just described demonstrate the advantages of using DMSO to trap HO' radicals with subsequent colorimetric determination of methane sulphinic acid. Of the previously-available molecular probes for determining HO', the aromatic compounds are the most convenient. When salicylates are used to trap HO', for example, the resulting hydroxylated aromatic derivatives can be detected by colorimetric [16], gas chromatographic [17], or fluorometric [19] methods. DMSO, as a trapping agent for HO', has the advantages of being soluble in both aqueous and organic solvents, of being non-toxic to biological systems [20-25] and of reacting very rapidly with HO' ($k = 7 \times 10^9$) [26] to yield a single hydroxylated product that has a larger molar extinction coefficient than that of the hydroxylated aromatic compounds. This method also requires less expensive instrumentation than gas chromatography and fluorescence spectroscopy.

Using this technique in the preliminary studies reported here, we found enhanced production of HO' for iron chelated with EDTA, NTA, and citrate, but low production for iron chelated with ADP-similar to results obtained by Baker and Gebicki [19]. Interestingly, the structures of the three chelators that most efficiently promoted HO' formation (EDTA, NTA, and citrate) all contain oxygen atoms in carboxyl groups that could chelate Fe^{+3}. Chelators that showed little promotion of hydroxyl radical formation (ADP, phytate, and phosphate buffer) all have phosphate groups available for chelation. Studies by Graf et al. [21] have indicated that the best promoters of Fenton chemistry are chelators that have a readily available coordination site, EDTA, NTA, and ADP. No free sites were reported for phytate and deferoxamine. Our studies of the iron chelates of EDTA, NTA, and deferoxamine support their hypothesis; however, we observed that ADP was no better than phytate in promoting hydroxyl radical generation. Sibille et al. [8] have suggested that the reason deferoxamine is so effective in blocking HO' formation is that the strong chelation at all six positions [29] prevents the reduction of Fe^{+3} to Fe^{+2} by O_2^- and that chelation by tyrosines in lactoferrin and transferrin similarly prevents Fe^{+3} reduction, whereas the purple acid phophastases permit the reduction.

In the case of EDTA-iron, the yield of HO' produced by the xanthine oxidase system was substantial and easily measured. However, the promotion of HO' formation by either EDTA or NTA is probably of little biological significance, since neither is found in vivo. With the exception of citrate, the hydroxyl radical generation found with the biologically available chelators tested in this study was approximately one tenth of that with EDTA-iron and was similar to that found with phosphate buffer. This lesser amount of HO' might still be important in a given setting, owing to the extreme reactivity and toxicity of hydroxyl radicals. Normal concentrations of non-protein bound iron in extracellular fluids have been estimated to be less than 5 µM [30] but may be higher in pathological conditions [31, 32]. The observation that 20 µM HO' was formed even at 10 µM Fe^{+3} in the presence of citrate is interesting, and suggests that citrate may have physiological significance as a promotor of Fenton chemistry.

Investigation of the iron chelators that may support the superoxide-driven Fenton reaction in complex biological systems deserves much greater attention. Although this reaction is often invoked in theoretical discussions of free radical mediated cellular injury, the importance of the chelator has not been emphasized by many authors [33-38]. If the superoxide driven Fenton reaction is to be confirmed as a pathophysiological mechanism, it is important that a physiologically plausible iron chelator that supports Fenton chemistry be identified.

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