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ASCORBATE AUTOXIDATION IN THE PRESENCE OF IRON AND COPPER CHELATES

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Chelates can inhibit the iron- and copper-catalyzed autoxidation of ascorbate at pH 7.0. Diethylenetriaminepentaacetic acid (DTPA or DETAPAC) and Desferal (deferoximane mesylate) slow the iron-catalyzed oxidation of ascorbate as effectively as reducing the trace levels of contaminating iron in buffers with Chelex resin. DETAPAC, EDTA and HEDTA (N-(2-hydroxyethyl)-ethylenediaminetriacetic acid) are effective at slowing the copper-catalyzed autoxidation of ascorbate while Desferal is ineffective. The ability to inhibit ascorbate autoxidation appears to parallel the rate of the reaction of superoxide with the iron chelate.

Key words: Ascorbate, Autoxidation, Iron Chelates, Copper Chelates

INTRODUCTION

It is now realized that metal ions, particularly iron and copper, catalyze many undesirable reactions in biological systems such as hydroxyl radical production by superoxide-generating systems[1,2,3,4], lipid peroxidation[5], autoxidation of catechols and catecholamines[6], mutagen formation during frying of beef[7], warmed-over flavor in cooked beef[8] as well as the autoxidation of ascorbate†[9,10,11,12]. In addition, the cytotoxicity of ascorbate in the presence of metal ions has been interpreted in terms of a "site specific" Fenton mechanism[13].

Scarpa *et al.*[14] have provided evidence for the production of superoxide anion by the autoxidation of ascorbate. They have proposed the production of one superoxide ion for each molecule of ascorbate oxidized. Their findings are in contrast to those of Halliwell and Foyer[15] who were unable to detect superoxide production in autoxidizing ascorbate systems. Koppenol and Butler[16] have demonstrated that the production of superoxide by autoxidizing ascorbate at pH 7 is not a thermodynamically favorable reaction. However, both products, $A^{\cdot -}$ and $O_2^{-\cdot}$ spontaneously disproportionate, thus the reaction may be pulled to the right.

† Ascorbate abbreviations: AH_2, ascorbic acid; AH^-, ascorbate monoion; $A^{\cdot -}$, ascorbate free radical; A, dehydroascorbic.

$$AH^- + O_2 \rightarrow A^{\cdot -} + O_2^{-\cdot} + H^+ \quad \Delta G^{\circ\prime} = +14.1 \text{ kcal}$$

The use of chelates to investigate the role of iron in many biological processes involving superoxide-generating systems has been very informative since its introduction[3]. It would appear that chelating agents may also be used as tools in determining the role of iron and copper in many other catalytic oxidation processes.

The effectiveness of various iron chelates as catalysts for ascorbate autoxidation has been examined at acid pH[10,11,12,17]. However, the nature of the metal chelate can vary considerably as the pH of the solution is altered[18]. I report here the inhibition of iron- and copper-catalyzed autoxidation of ascorbate by certain chelates at pH 7.0.

MATERIALS AND METHODS

Desferal (deferoxamine mesylate, often referred to as deferoxamine, desferoxamine or desferrioxamine) was from CIBA Pharmaceutical Co., Summit, NJ. Ascorbic acid was from Aldrich Chemical Co., Milwaukee, WI. All other reagents were from Sigma Chemical Co., St Louis, MO, and were used as received.

The ascorbate concentration was monitored by its absorbance at 265 nm. Absorbance measurements were done with a Perkin Elmer Lambda 5 spectrophotometer. The 50 mM phosphate buffer, pH 7.00 ± 0.04, was treated with Chelex 100 (Bio-Rad Laboratories, Richmond, CA) to remove transition metal impurities. Rather than pass the buffer solution through a prepared Chelex column, the Chelex 100 resin (100–250 mesh, sodium form) was added to the solution (≈ 5 ml/liter of buffer), the pH was adjusted and then allowed to stir overnight. The buffer was then filtered to remove the resin before use. Each chelating agent was present at 50 μM (neocuprion, 33 μM) and added iron or copper was present at 10 μM, introduced as $FeCl_3$ or $CuCl_2$. (Stock solutions were 0.60 mM $FeCl_3$ or $CuCl_2$ in 25 mM HCl.) The hemin solutions had no added iron. The hemin stock solution was prepared by dissolving the hemin in NaOH solution, pH 12. The order of reagent addition to the buffer was: chelating agent; metal solution; then ascorbate. The reactions were initiated by the addition of ascorbate at a final concentration of ≈ 0.1 mM, with an initial absorbance of 1.8 ± 0.1, at 265 nm. Lewin[9] reports a molar absorptivity for ascorbate at pH 6.1 of 25,400 ± 400 mol^{-1} cm^{-1} at 265 nm.

RESULTS

Trace levels of iron and other transition metals are present in buffer solutions at μM levels, unless care is taken to remove them. For example, 0.1 M KCl was found to contain iron at 2.5 μM[19], while 20 mM phosphate buffer has approximately 0.2 μM Fe with no detectable copper as seen by atomic absorption[20]. One popular method of removing trace metal ions is to pass the solution through a Chelex column. When ≈0.1 mM ascorbate was added to buffer which had been freshly "Chelexed", only a minimal loss of absorbance at 265 nm was observed, 0.5% in 15 minutes (< ≈2% in 60 minutes). However, if the ascorbate was added to freshly prepared phosphate buffer which had not been Chelexed, approximately 30% of the ascorbate was lost in 15 minutes. The addition of metal chelating agents to unchelexed buffer drastically slowed the loss of ascorbate, consistent with the presence of catalytic levels of metals being present in the buffer salts.

TABLE I

Additions to 50 mM pH 7.0 phosphate buffer	% Ascorbate lost in 15 minutes
Chelexed buffer	0.5
Unchelexed buffer	30
Unchelexed buffer + Desferal	1.7
Unchelexed buffer + DETAPAC	0.3
Unchelexed buffer + EDTA	3
Unchelexed buffer + HEDTA	1.5
Unchelexed buffer + Neocuprion	3
Chelexed buffer + Fe	3
Chelexed buffer + Hemin[a]	2
Chelexed buffer + Fe + Desferal	0.5
Chelexed buffer + Fe + DETAPAC	1
Chelexed buffer + Fe + EDTA	14
Chelexed buffer + Fe + HEDTA	7
Chelexed buffer + Fe + Neocuprion	4
Chelexed buffer + Cu (1 μM)	27
Chelexed buffer + Cu (2.5 μM)	85
Chelexed buffer + Cu (10 μM)	100
Chelexed buffer + Cu + Desferal	26
Chelexed buffer + Cu + DETAPAC	0.5
Chelexed buffer + Cu + EDTA	0.5
Chelexed buffer + Cu + HEDTA	0
Chelexed buffer + Cu + Neocuprion	4

[a]The hemin solution was 10 μM with no added iron.

The solutions were prepared in air-saturated 50 mM phosphate buffer, pH 7.00 ± 0.04. Each solution was 50 μM in chelating agent except neocuprion which was present at 33 μM due to its absorption at 265 nm. Cu^{2+} and Fe^{3+}, when added, were present at 10 μM, unless otherwise noted. All results above are the average of at least 3 separate experiments. In general the experiments repeated within 10% of the averages quoted here.

Chelating agents are able to alter the catalytic activity of transition metals. For example, DETAPAC and Desferal are able to greatly reduce the catalytic activity of iron in superoxide-generating systems[1,3,21,22]. As seen in Table I, chelating agents have a wide range of effects on the efficiency of iron and copper as catalysts for ascorbate autoxidation. DETAPAC and Desferal are as effective in reducing the catalytic activity of iron as removal by Chelex 100. EDTA and HEDTA are less effective in slowing the rate of iron-catalyzed autoxidation of ascorbate. However, EDTA, HEDTA and DETAPAC are very effective in slowing the copper-catalyzed autoxidation of ascorbate. The addition of 10 μM iron to Chelexed buffer is much less effective than addition of 10 μM copper. This may be partly due to the precipitation of the iron as an "$Fe(H_2O)_x(OH)_y$ gel"[23], reducing the surface area of the iron and consequently its efficiency as a catalyst.

DISCUSSION

Chelating agents have provided a wealth of information on the role of iron in the damage associated with superoxide-generating systems. Aust and White propose that iron chelators may be of clinical use in ameliorating tissue damage in ischemia[24].

Reoxygenation of ischemic tissue appears to result in excessive $O_2^-\cdot$ and H_2O_2 formation. These oxidants in the presence of iron can cause tissue damage. However, they have shown that the iron chelator deferoxamine can prevent tissue damage following ischemic anoxia. Chelating agents, particularly EDTA, have also been of great use as a sequestrant in foods to prevent oxidation[25] The report of Barnes and Weisburger[7] that iron plays a role in mutagen formation during the frying of beef and that EDTA reduces this formation, suggests that other chelating agents may be of value in modulating this food chemistry.

DETAPAC is much like EDTA in structure; however there appears not to be a water of coordination[26] as is known for EDTA[27]. This water of coordination is thought to be a key factor in the ability of Fe-EDTA to participate in the Haber-Weiss driven Fenton reaction, while Fe-DETAPAC and Fe-Desferal are non-participants[21,22,26].

The observation that Desferal is relatively ineffective in slowing the copper-catalyzed autoxidation of ascorbate parallels the findings of Gutteridge and Wilkins that Desferal was able to only partially inhibit the formation of a TBA-reactive product in an ascorbate-copper system[28]

Winterbourn and Sutton[29,20] have found that Fe(III)-DETAPAC at submicromolar levels is an effective catalyst for ·OH production in an H_2O_2-paraquat radical system. This is in contrast to the effects observed in superoxide-generating systems[21,22] and the observations here on ascorbate autoxidation. However, it is consistent with the greater reducing potential of the paraquat radical compared to $O_2^-\cdot$ and AH^-.

The results observed with hemin are probably explained by its insolubility in aqueous solutions. Aggregation would render it much less reactive as a catalyst for ascorbate oxidation. In addition it has been estimated that for an open monomeric heme such as hemin, the reduction potential is -1155 mV[30]. With the $A^{\cdot -}/AH^-$ couple having a reduction potential of -282 mV[16], the reduction of hemin by ascorbate is unlikely, and thus it is apparently unable to catalyze the oxidation of ascorbate.

The results of these experiments contrast with those reported by Martell[11] at pH 2.45. He reports that the rates for ascorbate autoxidation in the presence of iron chelators vary as:

$$\text{Rate (DETAPAC)} \approx 0.4 * \text{Rate (EDTA)} \approx 0.1 * \text{Rate (HEDTA)}.$$

While at pH 7.0 we find:

$$\text{Rate (DETAPAC)} \ll \text{Rate (EDTA)} \approx 2 * \text{Rate (HEDTA)}.$$

Thus the form the metal chelate takes as a function of pH plays a key role in its effectiveness as a catalyst.

It is interesting to note that the rate at which superoxide reacts with Fe(III)-EDTA is approximately twice the rate at which it reacts with Fe(III)-HEDTA[21]. In the ascorbate experiments it was found that roughly twice the ascorbate was lost in the presence of Fe(III)-EDTA as compared to Fe(III)-HEDTA. This parallel result suggests a similar mechanism for the reactions of superoxide and ascorbate with iron chelates.

These results on the effects of chelating agents on the ability of iron and copper to catalyze ascorbate autoxidation at pH 7.0 suggest that the effects of different chelating agents should be studied in various aspects of food chemistry and food preservation. In addition, they could provide information about the mechanism of a wide variety of human health problems ranging from cataract formation[31] to ischemia[24].

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