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SUPEROXIDE-DEPENDENT FORMATION OF HYDROXYL RADICALS FROM FERRIC-COMPLEXES AND HYDROGEN PEROXIDE: AN EVALUATION OF FOURTEEN IRON CHELATORS

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When a variety of ferric chelates are reacted with hydrogen peroxide in phosphate buffer deoxyribose is damaged and this damage is protected against by formate, thiourea and mannitol. Damage done by ferric complexes of citrate, EDTA, NTA, EGTA and HEDA is substantially inhibited by superoxide dismutase (SOD) whereas complexes of PLA, ADP and CDTA are moderately inhibited by SOD. The effects of SOD argue against hydrogen peroxide acting as a reductant in Fenton chemistry driven by ferric complexes and hydrogen peroxide. EDTA has proved to be a useful model for Fenton chemistry that is inhibited by SOD although, it is not unique in this respect.

KEY WORDS: Ferric complexes, SOD, hydroxyl, radical, EDTA, NTA, Fenton chemistry.

INTRODUCTION

Hydrogen peroxide (H_2O_2) is ascribed an important role in Fenton Chemistry as both a reductant (A) and oxidant (B) of iron salts. Reactions 'A' and 'B' lead ultimately to the formation of an agressive oxidant most frequently described as the hydroxyl radical ($^{\cdot}OH$) (For a review see Uri, 1952).

$$H_2O_2 + Fe^{3+} \rightarrow HO_2^{\cdot} + H^+ + Fe^{2+} \quad [A]$$

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad [B]$$

Complexes of iron with EDTA are frequently used to study the reaction of iron in Fenton chemistry since they provide highly soluble redox active forms of iron.[2–4] Superoxide ($O_2^{\cdot-}$) generated in the presence of ferric-EDTA reduces the iron complex to the ferrous state and provides H_2O_2 essential for $^{\cdot}OH$ formation.[3–6] Addition of superoxide dismutase to such a superoxide-driven Fenton reaction substantially inhibits formation of $^{\cdot}OH$ radicals. However, the products of the superoxide-driven reaction inhibited by superoxide dismutase are themselves able to generate an oxidising species. The reduction of iron and its reaction with H_2O_2 are shown above, for a simple iron salt, as equations [A] and [B]. When reaction [A] is studied using a ferric-EDTA complex further chemistry leading to the formation of an oxidising

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species is strongly inhibited by superoxide dismutase[7,8] suggesting that hydrogen peroxide does not reduce ferric EDTA as simply shown above in equation [A].[9] Hydroxyl radicals can be spin trapped in a mixture of ferric-EDTA and hydrogen peroxide.[9]

The present study was undertaken to see whether the inhibitory properties of superoxide dismutase towards the formation of ·OH radicals, and or other agressive oxidants, from a ferric chelate and hydrogen peroxide is seen when using chelators other than EDTA.

MATERIALS AND METHODS

Superoxide dismutase (bovine erythrocyte 2800 units/mg protein), catalase (bovine liver thymol free 14,100 units/mg protein), 2-deoxy D-ribose, albumin (bovine fatty acid free) was passed over a column of Chelex resin to remove copper associated with commercial batches. 2,2-dipyridyl (DP), bathophenanthroline sulphonate (BP), Ferrozine (FZ) Ferene S (FS), 1,10-phenanthroline (P), ethylenediamine-tetraacetic acid (EDTA), N-hydroxyethylethylenediamine-triacetic acid (HEDA), trans 1,2-diaminocyclohexane,N,N,N^1,N^1-tetraacetic acid (CDTA), ethyleneglycol-bis-(β-aminoethyl ether)N,N,N^1,N^1-tetraacetic acid (EGTA), diethylenetriaminepentaacetic acid (DETAPAC), picolinic acid (PLA), and nitrilotriacetic acid (NTA) were from the Sigma Chemical Company, St Louis, USA. Units of enzyme activity are those given in the Sigma catalogue. All other chemicals were obtained from Aldrich Chemicals, Milwaukee, USA.

1. Detection of oxidant damage to deoxyribose

A. Reaction of ferric complexes with hydrogen peroxide. Damage to deoxyribose as a method of detecting oxidant generation has been described in detail elsewhere.[10,11] The reaction mixture contained the following reagents at final concentrations stated: 27 mM sodium phosphate buffer pH 7.4 in 0.15 M NaCl. (For citrate and the free iron salt the buffer was as described above but at a pH value of 7.0), 2.7 mM deoxyribose, 0.02 mM ferric chloride (free or complexed as described) and 0.34 mM hydrogen peroxide. Inhibitors were added at the concentrations shown in appropriate Tables. The reaction was started by the addition of hydrogen peroxide.

Iron complexes were formed by pre-mixing freshly prepared ferric chloride with chelators in the following molar ratios: DP, BP, FZ, FS, P, Citrate, Picolinate (PLA) and ADP at 3 parts of chelator to 1 part of iron salt and EDTA, HEDA, CDTA, EGTA, DETAPAC and NTA at a 1:1 ratio. The iron complexes were used immediately before any self-reduction of the iron complex could occur. Ferric complexes and hydrogen peroxide were incubated at 37°C for 2 hours. After incubation 20 μl of catalase (1 mg/ml) was added to each tube to destroy any residual hydrogen peroxide and the tube contents incubated for a further 5 minutes at 37°C. Hydrogen peroxide can destroy TBA-reactive material and, heating deoxyribose with peroxide and iron complexes can also lead to increased radical damage to deoxyribose.

B. Reaction of ferrous complexes with oxygen. The reaction mixture contained 27 mM phosphate pH 7.4 in 0.15 M NaCl, 2.7 mM deoxyribose, 0.02 mM ferrous ammonium sulphate and chelators at the ratios described above. The ferrous salt was

not pre-mixed with the different chelators but added as the last reagent to start the reaction. Scavengers and inhibitors were added at the concentrations shown in appropriate Tables. Samples were incubated at 37° for 1 hour.

2. Thiobarbituric acid reactivity

After incubation of the reaction mixtures 0.5 ml of 1% (w/v) thiobarbituric acid in 0.05 M NaOH was added to each tube followed by 0.5 ml of 2.8% w/v trichloroacetic acid. The tube contents were heated at 100°C for 15 minutes. When cool, 0.05 ml of conc. HCL was added followed by 1.5 ml of butan-l-ol and the tube vigorously vortex mixed to extract the chromogen. Tubes were centrifuged at 3000 rpm for 7 minutes to separate the phases. The clear upper organic phase containing the TBA adduct was removed for measurement at 532 nm or fluorescence at 553 nm with excitation at 532 nm. Chromogens resulting from the reduction of ferric chelates of DP, BP, FS, FZ and P by thiobarbituric acid or reducing intermediates formed in the reaction did not extract into the butanol phase nor did they flouresce at 553 nm.

RESULTS

Ferric chloride and fourteen different complexes made with it stimulated the degradation of deoxyribose in the presence of hydrogen peroxide to release thiobarbituric acid-reactive material (Table 1). Measurable degradation was not seen in the absence

TABLE 1

The effect of superoxide dismutase and hydroxyl radical scavengers on deoxyribose degradation induced by ferric complexes and hydrogen peroxide.

	Fluorescent TBA reactivity	% Inhibition of Control value				
	Ex 532 nm Em 553 nm Units × 1	SOD (7 μg/ml)	Mannitol (53 mM)	Formate (53 mM)	Thiourea (7.0 mM)	Catalase (7 μg/ml)
Ferric chloride	100	48	66	75	66	87
DP ferric complex	55	24	44	32	81	81
BP ferric complex	43	33(S)	50	10	92	40
FZ ferric complex	70	35	66	58	66	89
FS ferric complex	115	21	73	65	57	90
P ferric complex	54	26(S)	28	11	94	77
CITRATE ferric complex	1,000	88	75	65	87	96
PLA ferric complex	98	61	68	69	68	82
ADP ferric complex	149	66	70	58	23	77
EDTA ferric complex	1,200	89	84	58	98	99
HEDA ferric complex	424	84	71	86	93	96
CDTA ferric complex	119	55	87	75	74	95
EGTA ferric complex	198	71	75	31	62	94
DETAPAC ferric complex	60	35	71	79	52	69
NTA ferric complex	760	83	68	78	91	95

(S) = stimulation of the reaction. Final reaction concentrations are shown. Iron concentrations 0.02 mM, hydrogen peroxide 0.34 mM. Fluorescence units shown are values less the blank (40 units). 610 fluorescence units were equal to an absorbance value of 0.146 at 532 nm. SOD = Superoxide dismutase. The results shown are the mean of 3 or more separate experiments that differed by less than ± 5%.

TABLE 2
Protective action of Albumin on deoxyribose degradation by Ferric-EDTA and hydrogen peroxide.

Ferric-EDTA + H_2O_2 (Control value = 1200 units)	% Inhibition by: ALBUMIN	SOD
7 μg/ml Protein	8%	89%
14 μg/ml Protein	32%	89%
28 μg/ml Protein	40%	89%
42 μg/ml Protein	55%	89%

The albumin sample was passed over Chelex resin to remove copper ions associated with commercial preparations.

of added hydrogen peroxide or iron. The most reactive of these complexes under the conditions studied were citrate, EDTA, HEDA and NTA. Addition of sueproxide dismutase showed that only the reaction in the presence of EDTA, citrate, NTA, HEDA and EGTA was substantially inhibited by the enzyme (Table 1) although, moderate inhibition was also seen in the presence of ADP, CDTA and PLA. As expected, catalase almost completely inhibited damage in all reactions except that of the chelator BP (Table 1). Albumin added as a control for non-specific radical scavenging was without significant effect at a concentration of 7 μg/ml. However, as the concentration was increased so albumin became more inhibitory (Table 2). Addition of three chemically distinct but commonly used scavengers of the hydroxyl radical namely, mannitol, formate and thiourea to the ferric complex – H_2O_2 reaction showed that, with few exceptions, all were generally good inhibitors of damage to deoxyribose (Table 1). Formate poorly protected in the two reactions stimulated by SOD. Similar patterns of inhibition were seen with the non-complexed iron salt. Urea added as a control for non-specific radical scavenging was without effect (data not shown).

TABLE 3
The effect of hydroxyl radical scavengers on deoxyribose degradation induced by ferrous complexes.

	TBA reactivity	% Inhibition of Control value		
	A 532 nm	Mannitol (53 mM)	Formate (53 mM)	Thiourea (2.7 mM)
Ferrous ammonium sulphate	0.138	91%	57%	88%
DP ferrous complex	0.144	95%	55%	56%
BP ferrous complex	0.034	78%	51%	88%
FZ ferrous complex	0.197	95%	50%	77%
FS ferrous complex	0.318	94%	56%	77%
P ferrous complex	0.048	61%	64%	44%
EDTA ferrous complex	0.107	94%	72%	80%
HEDA ferrous complex	0.069	87%	98%	73%
CDTA ferrous complex	0.076	94%	89%	93%
EDTA ferrous complex	0.293	96%	54%	78%
DETAPAC ferrous complex	0.085	92%	78%	46%
CITRATE ferrous complex	0.078	96%	90%	81%
NTA ferrous complex	0.077	66%	84%	74%
ADP ferrous complex	0.128	99%	60%	76%
PLA ferrous complex	0.128	98%	67%	78%

Final reaction concentrations are shown. Iron concentration 0.02 mM. The results shown are a mean of 3 or more separate experiments which differed by less than ± 5%.

When ferric complexes were replaced by ferrous complexes (and hydrogen peroxide omitted from the reaction), deoxyribose damage was greater in most cases, with ferrous-Ferene S and ferrous-EDTA being the most reactive (Table 3). Similar patterns of inhibition were seen with mannitol formate and thiourea. Urea added as a non-specific control was without effect (data not shown).

DISCUSSION

A variety of ferric chelates prepared freshly from a solution of ferric chloride was able to react with hydrogen peroxide in phosphate buffer at pH 7.4 to produce an oxidant species able to degrade the carbohydrate deoxyribose. For most, the reaction was extremely weak and the product of deoxyribose degradation could only be measured spectrofluorimetrically. However, EDTA, citrate and NTA were reactive giving absorbance values suitable for spectrophotometric measurement. In classical Fenton chemistry hydrogen peroxide is widely ascribed a dual role as both a reductant and oxidant of iron salts and their complexes. For certain iron chelators such as bleomycin, hydrogen peroxide has been shown not to reduce the iron bound to it[12] and it has recently been argued that iron bound to EDTA is also unlikely to be reduced by hydrogen peroxide.[9] If EDTA complexed iron were reduced by hydrogen peroxide the subsequent reactions leading to the formation of hydroxyl radicals should not be inhibited by the enzyme superoxide dismutase.[9] Data presented here from fourteen different iron chelators suggests that the superoxide dismutase inhibitable reaction, of ferric chelates with hydrogen peroxide, leading to hydroxyl radical formation is common to several chelators other than EDTA. If the reduction of a ferric-EDTA complex by hydrogen peroxide did take place, as depicted for a simple iron salt in equation [A], then we might expect that some 50% of the ferrous ions required for Fenton chemistry could be provided by superoxide-dependent reduction of ferric ions (shown in equation A as its protonated form $HO_2^{\cdot}$). This would suggest that superoxide dismutase might inhibit the ultimate formation of hydroxyl radicals by up to 50%. Inhibitions approaching this value were obtained with the chelator CDTA and the free iron salt although, so far, there is no confirmatory evidence to verify reduction by hydrogen peroxide. For other chelators poor inhibition seen with superoxide dismutase may equally be used to support the assumption that hydrogen peroxide does not reduce the ferric complex.

Mannitol and thiourea are known to form complexes with metal ions and hydrogen peroxide[13-16] as well as to scavenge hydroxyl radicals.[17] However, it is unlikely that mannitol, thiourea or the detector molecule deoxyribose would be able to compete for metal binding with most of the chelators studied here. Primary radical generation would be expected to take place on, or close to, the ferric complex and deoxyribose will be damaged by reactive radicals released into free solution from the iron chelate. Alternatively a ternary complex may form between the iron complex, phosphate and detector molecule.[18,19]

When hydrogen peroxide is added to the ferric complexes abundant peroxide is present in the reaction during the two hour incubation period and excess is removed by catalase to terminate the reaction. In the presence of formate and thiourea, but not in the presence of mannitol, bathophenanthroline and ferrozine produced chromogenic ferrous complexes during incubation suggesting that reducing intermediates were formed from thiourea and formate.

The widely used metal chelator EDTA when complexed to iron and participating in Fenton chemistry appears to release hydroxyl radicals into free solution where they can be scavenged according to their known second order rate constants for various molecules (radiomimetic effect).[20] This occurs with EDTA when ferrous salts are used alone[20] when ferric salts, ascorbate and hydrogen peroxide are present[21] or when ferric salts plus hydrogen peroxide are used.[9] In the latter case evidence for the generation of hydroxyl radicals has been obtained using EPR spin trapping experiments.[9] Recent detailed studies with the chelators dipyridyl and EDTA,[9] using higher chelator concentrations and lower scavenger concentrations, suggest that complexed ferryl (FeO^{2+}) ions may sometimes be intermediates in the formation of hydroxyl radicals. Reactive iron intermediates such as these may have different stabilities, depending on the iron-binding ligand, which determines their survival and reactivity towards the detector molecule. Evidence presented here, however, even for a simple iron salt, strongly suggests that hydroxyl radicals are the major damaging species formed by most iron complexes reacting with hydrogen peroxide.

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