SUPEROXIDE-DEPENDENT FORMATION OF HYDROXYL RADICALS IN THE PRESENCE OF IRON SALTS

Its role in degradation of hyaluronic acid by a superoxide-generating system

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Received 5 October 1978

1. Introduction

The superoxide radical, $O_2^{\cdot-}$, is formed in all aerobic cells and has many deleterious effects [1,2]. Polymorphonuclear leucocytes produce $O_2^{\cdot-}$ during ingestions of bacteria [3]. In several inflammatory conditions, leucocytes enter the joints of the body [4]. Since synovial fluid has little superoxide dismutase activity, the $O_2^{\cdot-}$ released by the leucocytes is not detoxified and reacts with joint components, causing damage. In particular, hyaluronic acid is depolymerised and the synovial fluid loses its lubricating properties [4]. McCord [4] showed that this depolymerisation could be mimicked by exposing pure hyaluronic acid solutions or synovial fluid itself to a mixture of xanthine and xanthine oxidase, which generates $O_2^{\cdot-}$. He found that depolymerisation could be prevented not only by addition of superoxide dismutase but also by catalase or by mannitol, a scavenger of the hydroxyl radical ($^{\cdot}OH$). Since the highly-reactive $^{\cdot}OH$ radical attacks and destroys carbohydrates rapidly [5], he suggested that depolymerisation was due to formation of $^{\cdot}OH$ by a direct reaction between $O_2^{\cdot-}$ and H_2O_2 (the Haber-Weiss reaction) [4] – eq. (1):

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + {}^{\cdot}OH + OH^- \qquad (1)$$

Subsequent studies have shown that reaction (1) does not proceed at significant rates in biochemical systems (for a list of references, see [6]). However, addition of small amounts of iron salts to superoxide-generating systems causes formation of $^{\cdot}OH$, probably by reactions (2) and (3) below [6–8]:

$$Fe^{3+}\text{-compound} + O_2^{\cdot-} \longrightarrow Fe^{2+}\text{-compound} + O_2 \qquad (2)$$

$$Fe^{2+}\text{-compound} + H_2O_2 \longrightarrow Fe^{3+}\text{-compound} + {}^{\cdot}OH + OH^- \qquad (3)$$

$$\text{Net: } O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + OH^- \qquad (1)$$

This 'iron-catalysed Haber-Weiss reaction' has been suggested by many authors to be the mechanism by which $^{\cdot}OH$ can be formed in biochemical systems (for a list of references see [6]). Reactions (2) and (3) can be inhibited by the iron chelator diethylenetriaminepentaacetic acid (DETAPAC), which should therefore be a useful tool in probing the involvement of these reactions in some of the many systems in which $^{\cdot}OH$ appears to be formed from $O_2^{\cdot-}$ [6,8]. In this paper, I show that the iron chelator 4,7-diphenyl-1,10-phenanthroline sulphonate (bathophenanthroline sulphonate, abbreviated BPS) also inhibits reactions

(2) and (3). The effects of DETAPAC and BPS on $O_2^{\cdot-}$-induced degradation of hyaluronic acid are reported.

2. Materials and methods

2.1. *Reagents*

Superoxide dismutase (EC 1.15.1.1) from bovine blood, hyaluronic acid type III-P, BPS, DETAPAC and the sodium salt of xanthine were obtained from Sigma Chemical Corp. Catalase (EC 1.11.1.6), free of superoxide dismutase [9], and xanthine oxidase were obtained from Boehringer Corp.

2.2. *Assays of enzymes*

Xanthine oxidase activity was measured by the uptake of O_2 using an O_2-electrode [6]. Catalase was measured by the fall in $A_{240\ nm}$ as H_2O_2 is destroyed: 1 unit catalyses degradation of 1 μmol H_2O_2/min under the conditions in [10]. Superoxide dismutase was assayed by the cytochrome *c* method [11]: one unit inhibits cytochrome *c* reduction by 50%.

2.3. *Measurement of hydroxylations*

Hydroxylation of salicylate by the xanthine–xanthine oxidase system was determined as in [6]. Hydroxylation of salicylate by high concentrations of Fe^{2+}–EDTA was determined by a modification of [12]. Reaction mixtures contained in 1 ml total vol.: 5 μmol salicylic acid; 100 μmol KH_2PO_4; 20 μmol EDTA; 5 μmol $FeSO_4$; and sufficient KOH to adjust the whole reaction mixture to pH 7.2. After incubation at 25°C for 1 h, 20 μl conc. HCl was added, the reaction products were extracted into ether and measured as in [13] except that the final absorbance was read immediately after addition of the KOH.

2.4. *Measurement of hyaluronic acid degradation*

Degradation was determined by the decrease in viscosity of a 3 ml reaction mixture incubated with shaking at 25°C for 30 min as in [4]. Each determination was carried out in triplicate and the effect of each reagent was determined in at least four different experiments using different batches of hyaluronic acid.

3. Results

3.1. *Inhibition of the iron-catalysed Haber-Weiss reaction*

$O_2^{\cdot-}$-dependent formation of $^{\cdot}OH$ radicals can easily be detected by the ability of such radicals to hydroxylate aromatic compounds [6]. Table 1 shows that hydroxylation of salicylate by the xanthine–xanthine oxidase system occurs if traces of iron salts are present. Hydroxylation is inhibited by superoxide dismutase, by catalase and by scavengers of $^{\cdot}OH$ [6]. Addition of EDTA increases the rate of hydroxylation, since Fe^{2+}–EDTA complexes react more rapidly with $O_2^{\cdot-}$ than do free iron salts [14]. However, low concentrations of DETAPAC or BPS inhibit hydroxylation (table 1). Neither compound affects the assay used to measure hydroxylation and DETAPAC has no effect on xanthine oxidase activity. Incubation of xanthine oxidase with 1 mM BPS for 30 min caused a 35% inhibition of the enzyme, which is too small to account for the inhibitory effects of BPS on hydroxylation (table 1). Further, BPS still inhibited hydroxylation when the assay was carried out in the presence of a large excess of xanthine oxidase, so that the rate of hydroxylation was independent of the amount of enzyme present.

Hydroxylation of aromatic compounds can be achieved in the absence of an $O_2^{\cdot-}$-generating system if a very high concentration of iron salt is present, especially if it is chelated to EDTA [12]. Here, $O_2^{\cdot-}$ appears to be generated by autoxidation of the iron salt and dismutates to H_2O_2, which then reacts with the iron salt to form $^{\cdot}OH$ [15]. Table 2 shows that BPS and DETAPAC could substantially decrease formation of $^{\cdot}OH$ in this system. Hydroxylation was also inhibited by scavengers of $^{\cdot}OH$ (e.g., mannitol, formate, ethanol, thiourea) but not by KCl or urea, which do not react with $^{\cdot}OH$ at significant rates (data not shown). Hence BPS and DETAPAC seem to be general inhibitors of the formation of $^{\cdot}OH$ radicals in the presence of iron salts.

3.2. *Effect of inhibitors on the degradation of hyaluronic acid*

Depolymerisation of hyaluronic acid was easily demonstrated in the presence of a xanthine–xanthine oxidase system, in agreement with [4]. Loss of viscosity was almost completely prevented by catalase

Table 1
Effect of chelating agents on hydroxylation of aromatic compounds by the xanthine–xanthine oxidase Fe^{2+}–EDTA system

Addition to reaction mixture	Final conc. of addition	EDTA present	nmol diphenol produced/h	% rate of reaction
None	–	Yes	110	100
DETAPAC	1.5	Yes	65	59
	3.0	Yes	40	36
BPS	0.3	Yes	69	63
	0.6	Yes	60	55
	1.0	Yes	42	38
None	–	No	64	100 (58)[a]
DETAPAC	0.5	No	39	61
	1.0	No	30	47
	1.5	No	25	39
BPS	0.3	No	37	58
	0.6	No	24	38
	1.0	No	12	19

[a] Result expressed as % of the rate in the presence of EDTA. The results of a typical experiment are presented, but they were highly reproducible

Assays of salicylate hydroxylation in the presence of 100 μM $FeSO_4$ were conducted as in [6]. Where indicated, 300 μM EDTA was also present. Neither DETAPAC nor BPS affect the assay used to measure diphenols [6]

Table 2
Effect of chelating agents on hydroxylation of aromatic compounds by the $FeSO_4$–EDTA–phosphate system

Chelator added	$\frac{[\text{chelator}]}{[\text{EDTA}]}$	nmol diphenol produced/h	% rate of hydroxylation
None	–	550	100
None (–salicylate)	–	0	0
None (–$FeSO_4$)	–	0	0
None (–EDTA)	–	100	18
DETAPAC	1.0	363	66
	1.5	167	30
EDTA replaced by equal conc. DETAPAC	–	215	39
BPS	0.2	500	91
	0.5	278	51
	1.0	40	7
EDTA replaced by equal conc. BPS	–	0	0

Hydroxylation was measured as described in section 2. All reaction mixtures contained EDTA unless otherwise stated. Neither DETAPAC nor BPS affect the assay used to measure hydroxylation. The results of a typical experiment are presented, but they were highly reproducible

Table 3
Effect of iron chelators and other reagents on degradation of hyaluronic acid by the xanthine–xanthine oxidase system

Reagent added	Final conc. reagent added	$FeSO_4$ present	% hyaluronate originally present, degraded during reaction
None	–	No	75
None (–xanthine)	–	No	0
None (–xanthine oxidase)	–	No	0
Superoxide dismutase	100 units/3 ml	No	21
Catalase	4000 units/3 ml	No	8
Mannitol	16.7 mM	No	46
	30 mM	No	22
Sodium formate	16.7 mM	No	27
	30 mM	No	14
Bovine serum albumin	0.5 mg/3 ml	No	74
DETAPAC	1.0 mM	No	42
	2.0 mM	No	32
BPS	1.0 mM	No	44
EDTA	1.0 mM	No	72
None	–	Yes	91
None (–xanthine oxidase)	–	Yes	0
DETAPAC	1.0 mM	Yes	72
	2.0 mM	Yes	60
Superoxide dismutase	100 units/3 ml	Yes	27
Catalase	4000 units/3 ml	Yes	26
Bovine serum albumin	0.5 mg/3 ml	Yes	90
BPS	1.0 mM	Yes	65

Degradation of hyaluronic acid was determined by the decrease in viscosity after incubation as described in section 2. A typical set of results is presented, but they were highly reproducible. Where indicated, reaction mixtures contained 0.2 μmol/3 ml $FeSO_4$. Units of catalase and superoxide dismutase were as described in section 2. Increasing the amount of xanthine oxidase present in the reaction mixture to allow for the slight inhibitory effect of BPS on this enzyme (see text) did not alleviate the inhibition of hyaluronate degradation by BPS

or by superoxide dismutase, but not by bovine serum albumin at equal or greater protein concentrations (table 3). The ·OH scavengers mannitol and formate also inhibited strongly.

If formation of ·OH in this system is due to an iron-catalysed Haber-Weiss reaction (eq. (2), (3)), then DETAPAC and BPS should inhibit. This was found to be the case (table 3). The extent of inhibition that could be achieved was similar to that seen in the model systems for ·OH formation (tables 1, 2). In contrast, EDTA did not inhibit. Addition of traces of $FeSO_4$ accelerated hyaluronate depolymerisation by the xanthine–xanthine oxidase system: the increased rate was again inhibited by catalase, superoxide dismutase, BPS, DETAPAC and scavengers of ·OH radicals. Addition of EDTA plus $FeSO_4$ did not increase the rate of degradation above that in the presence of $FeSO_4$ alone. $FeSO_4$ did not itself cause any degradation of hyaluronic acid (table 3).

4. Discussion

The iron-catalysed Haber-Weiss reaction has been demonstrated in 3 model systems [6–8]. Results to date show that DETAPAC inhibits 2 of these, namely

the hydroxylation assay used here and a spin-trapping assay [6,8]. In this paper, BPS has also been shown to be inhibitory.

Both DETAPAC and BPS inhibit degradation of hyaluronic acid by the xanthine–xanthine oxidase system, whereas EDTA does not. The simplest explanation of this observation is that reactions (2) and (3) are providing the ·OH radicals needed for the degradation. It might be expected, by comparison with the model system in table 1, that EDTA should accelerate degradation. However, it may well be that the iron present is so firmly attached to the carboxyl groups of the hyaluronic acid that it cannot be removed by the EDTA. Even when extra $FeSO_4$ was added, which increased the rate of degradation, subsequent addition of EDTA did not further increase degradation.

Neither DETAPAC nor BPS reacts with $O_2^{\cdot-}$. An alternative explanation of their effects would be that they are scavengers of ·OH. If this were the case, however, their rate constants would have to be extremely high, since they are effective at much lower concentrations than such powerful ·OH scavengers as mannitol and formate. It is also difficult to see why EDTA, which has a structure similar to that of DETAPAC, should have a completely different effect. Also, DETAPAC (1–3 mM) does not inhibit hydroxylation by the NADH/phenazine methosulphate system (data not shown), which is due to formation of ·OH from H_2O_2 [17].

The results presented here may have medical significance. It is likely that the hyaluronic acid in synovial fluid contains even more metal ions than does the purified acid used here [4]. Chronic joint inflammations have sometimes been treated by injection of superoxide dismutase into the joint [16]. Although this is beneficial, the use of a pure protein is expensive. A simple inhibitor of the iron-catalysed Haber-Weiss reaction should, if found suitable for clinical use, achieve the same effect at much lower cost.

Acknowledgement

I thank the Central Research Fund of the University of London for financial support.

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