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

Is it a mechanism for hydroxyl radical production in biochemical systems?

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1. Introduction

The superoxide radical, $O_2^{\cdot-}$, is formed in all aerobic organisms and has many deleterious effects [1,2]. There is considerable evidence that in the presence of H_2O_2, $O_2^{\cdot-}$ can give rise to the even-more-toxic hydroxyl radical, $^{\cdot}OH$ [1–3]. In 1934, Haber and Weiss [4] proposed that $O_2^{\cdot-}$ and H_2O_2 react together to produce $^{\cdot}OH$ directly, as shown in eq. (1):

$$H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + {}^{\cdot}OH + OH^- \quad (1)$$

The Haber-Weiss reaction was therefore naturally proposed as the source of $^{\cdot}OH$ generated from $O_2^{\cdot-}$ in biochemical systems [1]. Unfortunately, although this reaction is thermodynamically possible [5], several attempts to demonstrate it have failed [6–8] and it is now generally agreed that it does not occur at significant rates. Traces of chelated metal ions are present in all biochemical systems, however, and several authors have proposed that such chelates could catalyse reaction (1) [9–12]. For example, iron chelates react rapidly with $O_2^{\cdot-}$ [13] and one can envisage a mechanism such as the following for generation of $^{\cdot}OH$ radicals

$$Fe^{3+}\text{-chelate} + O_2^{\cdot-} \rightarrow O_2 + Fe^{2+}\text{-chelate} \quad (2)$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \rightarrow Fe^{3+}\text{-chelate} + {}^{\cdot}OH + OH^- \quad (3)$$

Net reaction: $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$ (1)

McCord and Day [14] demonstrated the feasibility of reactions (2) and (3) by using the ability of tryptophan to react with $^{\cdot}OH$ (with loss of absorbance at 278 nm) to demonstrate formation of $^{\cdot}OH$ by a $O_2^{\cdot-}$-generating system to which iron chelates had been added. However, the absorbance changes obtained were small and our attempts to investigate this system further have been hampered by absorbance changes due to the iron chelates themselves [15]. Hydroxyl radicals can be detected more sensitively by their ability to hydroxylate aromatic compounds [3,16] and, in an attempt to confirm [14], I have studied hydroxylation of such compounds by a $O_2^{\cdot-}$-generating system. $O_2^{\cdot-}$ itself is insufficiently reactive to hydroxylate aromatic compounds [17]. The results presented below confirm that reactions (2) and (3) can occur and they also show that they can be inhibited by the iron chelator diethylene-triamine-pentaacetic acid (DETAPAC). This chelator should therefore be an extremely valuable probe of the involvement of an 'iron-catalysed Haber-Weiss reaction' in biochemical systems, and the results of its use in one such system are presented.

Abbreviation: DETAPAC, diethylenetriamine pentaacetic acid

2. Materials and methods

Catalase was obtained from Boehringer Corp., London W5; it was completely free of superoxide dismutase activity [18]. Erythrocuprein prepared according to the method in [19], horse-spleen ferritin, xanthine (sodium salt), DETAPAC and horseradish peroxidase (type VI) (EC 1.11.1.7) were purchased from Sigma Chemical Corp. Peroxidase concentration was calculated from A_{403} ($E_{403} = 10^5\ M^{-1}cm^{-1}$ [20]). Myeloperoxidase (EC 1.11.1.8) was prepared from granulocytes [21]. Where indicated, enzymes were denatured by heating at 100°C for 20 min and cooling before use. Xanthine oxidase was obtained from Boehringer or from Sigma.

2.1. *Enzyme assays*

Superoxide dismutase (EC 1.15.1.1) was assayed by the cytochrome *c* method: 1 unit inhibits cytochrome *c* reduction by 50% under the assay conditions in [19]. Catalase (EC 1.11.1.6) was assayed by the fall in A_{240} as H_2O_2 was destroyed; the assay conditions were as in [22] except that 50 mM KH_2PO_4–KOH buffer, pH 7.4, was the buffer used in the reaction mixture. One unit of catalase is that amount which catalyses the breakdown of 1 μmol H_2O_2/min under these conditions. Xanthine oxidase (EC 1.2.3.2) was assayed by measuring urate formation at 290 nm or by measuring O_2 uptake using a Hansatech O_2 electrode (Hansatech Ltd., King's Lynn) calibrated according to the manufacturer's instructions. Reaction mixtures at 25°C contained, in final vol. 2.4 ml, 1 ml saturated solution of xanthine in water and 150 μmol KH_2PO_4, adjusted to pH 7.4 with KOH. Reactions were started by adding 20 μl xanthine oxidase.

2.2. *Assay of hydroxylation by iron/xanthine/xanthine oxidase system*

Reaction mixtures contained, in final vol. 1.00 ml, 2.5 μmol salicylic acid, iron salt and EDTA in the amounts stated in the text, 0.7 ml saturated solution of the sodium salt of xanthine in 0.15 M KH_2PO_4 buffer and sufficient KOH to adjust the whole reaction mixture to pH 7.4. The iron salt and EDTA (or DETAPAC) were always mixed together immediately before addition of the other reagents. Reactions were initiated by adding 20 μl xanthine oxidase (Boehringer or Sigma preparations gave identical results) followed by incubation at 25°C for 1 h. Then 10 μl conc. HCl was added. Reaction products were extracted into ether and assayed for diphenols as in [3].

3. Results

When aromatic compounds were incubated with xanthine and xanthine oxidase, at pH 7.4, no formation of hydroxylated products was detected. However, addition of low concentrations of $FeSO_4$ or $FeCl_3$ in the presence of an excess of EDTA caused significant hydroxylation. Table 1 shows some typical results using salicylate as phenol. Formation of diphenolic products from this substrate was completely prevented by omitting xanthine, xanthine oxidase, iron salt, or salicylate itself from the reaction mixtures. Omission of EDTA reduced the amount of hydroxylation. Addition of higher concentrations of Fe^{2+}-EDTA increased the rate of hydroxylation, but at such concentrations there was significant hydroxylation in the absence of xanthine oxidase (table 1). This presumably represents the hydroxylation of aromatic compounds in the presence of high concentrations of metal ions reported in [23]. When allowance was made for it, it could be seen that hydroxylation by the iron/xanthine/xanthine oxidase system reached a maximum rate at ~ 100 μM $FeSO_4$ or $FeCl_3$ in the presence of an excess of EDTA. The rate of hydroxylation was linear with time for ~ 60 min, and then began to decline. Hence most studies were carried out using a 1 h incubation period in the presence of 100 μM iron salt and 300 μM EDTA.

Hydroxylation by the iron/xanthine/xanthine oxidase system could be completely inhibited by small amounts of catalase or superoxide dismutase (table 2). Heat-denatured enzyme or bovine serum albumin added at equal or greater protein concentrations had no effect. These results show that both superoxide and H_2O_2 are needed for hydroxylation to occur. Addition of H_2O_2 (tested in the range 0.02–0.2 mM) to the reaction mixtures did not stimulate hydroxylation by the iron/xanthine/xanthine oxidase system when allowance was made for the fact that H_2O_2 induced some hydroxylation in the absence of xanthine oxidase (presumably by forming a 'Fenton' system with the iron salt present).

Table 1
Hydroxylation of salicylate by xanthine/xanthine oxidase in the presence of iron–EDTA

Iron salt added	Conc. (μM)	nmol diphenol produced/h		
		xanthine oxidase present	xanthine oxidase omitted	net hydroxylation in presence of xanthine oxidase
$FeSO_4$	0	0	0	0
	10	30	0	30
	20	51	0	51
	50	71	0	71
	100	92	1	91
	200	135	45	90
	300	155	56	99
	100 (omit EDTA)	36	0	36
	100 (omit salicylate)	0	0	0
	100 (omit xanthine)	0	0	0
$FeCl_3$	100	95	2	93

Reaction conditions were as described in section 2; the iron salts were added at the concentration stated, together with 3-times the concentration of EDTA (e.g., for 200 μM $FeSO_4$, 600 μM EDTA was added) unless otherwise stated

Table 2
Effects of superoxide dismutase and catalase on hydroxylation by xanthine/xanthine oxidase in the presence of iron–EDTA

Iron salt added	Inhibitor added	nmol diphenol produced/h	% Inhibition
$FeSO_4$	None	109	0
	Superoxide dismutase		
	10 units	63	42
	20 units	51	53
	50 units	44	60
	100 units	11	90
	Heated superoxide dismutase		
	100 units[a]	108	1
	Catalase		
	18 units	55	50
	36 units	32	71
	93 units	11	90
	150 units	0	100
	Heated catalase		
	150 units[a]	110	0
$FeCl_3$	None	110	0
	Superoxide dismutase		
	100 units	17	85
	Catalase		
	150 units	0	100

[a] Activity before heating the enzyme

Reaction mixtures were as described in section 2; they contained 100 μM iron salt and 300 μM EDTA

Table 3
Effect of hydroxyl radical scavengers on hydroxylation by the xanthine/xanthine oxidase system in the presence of Fe^{2+}–EDTA

Scavenger added	Final conc. (mM) in reaction mixture	nmol diphenol produced/h	% Inhibition of hydroxylation	Rate constant for scavenger reaction with ·OH ($M^{-1}s^{-1}$)
None	–	102	0	–
Mannitol	5	64	37	1.0×10^9
	10	50	51	
	25	36	65	
	47	15	85	
Sodium formate	5	49	52	2.7×10^9
	10	41	60	
	25	34	67	
	47	7	93	
Thiourea	1	57	44	4.7×10^9
	2	44	57	
	5	24	76	
KCl	50	104	0	$<10^3$
Urea	5	102	0	$<7 \times 10^5$

Reaction mixtures were as described in section 2; they contained 100 μM $FeSO_4$ and 300 μM EDTA. Similar results were obtained when $FeCl_3$ replaced $FeSO_4$. None of the above scavengers affected the assay for diphenol production. Neither formate nor mannitol affected xanthine oxidase activity. Thiourea inhibited xanthine oxidase slightly as assayed by O_2 uptake (final conc. 5 mM inhibited by 15%, 8 mM by 24% and 16.7 mM by 40%). These inhibitions are clearly too small to account for more than a small part of the striking effect of thiourea on hydroxylation. Rate constants were taken from the compilation in [24]

Hence sufficient H_2O_2 must be generated in the reaction mixture.

Since both $O_2^{\cdot-}$ and H_2O_2 are essential, they may be reacting together to form the hydroxyl radical, which is the true hydroxylating agent. In agreement with this, scavengers of ·OH were found to inhibit hydroxylation by the iron/xanthine/xanthine oxidase system (table 3). The amount of scavenger required to inhibit was qualitatively correlated with its rate constant for reaction with ·OH. Urea and KCl, which react poorly, if at all, with ·OH radicals, had little effect.

Attempts were made to replace iron salts with other metal ions and complexes. No hydroxylation by the xanthine/xanthine oxidase system could be detected when iron was replaced by the following metal ions, each tested at final conc. 200 μM both in the presence and in the absence of 600 μM EDTA: copper (II) sulphate; zinc (II) sulphate; nickel (II) chloride; chromium (II) chloride; manganese (II) chloride; cobalt (III) sulphate; vanadyl sulphate; or ceric ammonium sulphate. Similarly, addition of horseradish peroxidase or myeloperoxidase to final conc. 0.09 μM did not promote hydroxylation, nor did 10^2–10^3 μg horse-spleen ferritin.

3.1. *Effect of chelating agents*

Buettner et al. [25] have claimed that the iron chelator DETAPAC appeared to prevent formation of hydroxyl radicals, identified by a spin-trapping technique, in the xanthine/xanthine oxidase system. This claim is supported by the results in table 4, which show that DETAPAC could not replace EDTA in promoting hydroxylation by the iron/xanthine/xanthine oxidase system. They also show that an excess of DETAPAC inhibited hydroxylation even

Table 4
Effect of diethylenetriamine pentaacetic acid (DETAPAC) on hydroxylation by xanthine/xanthine oxidase in the presence of iron salts

Reaction mixture (μM)		nmol diphenol produced/h	% inhibition of hydroxylation
EDTA	DETAPAC		
300	0	95	0
0	0	41	57
0	300	29	69
300	300	72	24
300	730	65	32
300	1400	40	58

Reaction mixtures were as described in section 2; each contained 100 μM $FeSO_4$ and the chelating agents at the concentrations stated. DETAPAC had no effect on xanthine oxidase nor on the assay used to measure hydroxylation

when EDTA was present, presumably by competing for the available iron salt.

Since DETAPAC inhibits the 'iron-catalysed Haber-Weiss reaction' (table 4), it is potentially very useful in testing whether or not this reaction accounts for formation of 'OH radicals in biochemical systems. One such system is the hydroxylation of aromatic compounds by horseradish peroxidase in the presence of dihydroxyfumarate. Hydroxylation by this system is completely inhibited by superoxide dismutase and by scavengers of 'OH [3], but I have found that DETAPAC, tested at conc. $\leqslant$ 3 mM, had no effect on hydroxylation of salicylate or 4-hydroxycinnamate by this system at pH 6 under the assay conditions in [3].

4. Discussion

The results presented in this paper show that an iron-catalysed Haber-Weiss reaction is feasible, i.e., reactions (2) and (3) can occur and give rise to $O_2^{\cdot-}$- and H_2O_2-dependent formation of 'OH radicals. This supports the suggestions [2,9–12] and experiments [12,14,25]. It is noteworthy that other metals tested could not replace iron, nor could physiological iron complexes such as peroxidases and ferritin.

It must now be decided whether reactions (2) and (3) are the true mechanism by which 'OH is generated from $O_2^{\cdot-}$ and H_2O_2 in biochemical systems. In this paper the iron chelator DETAPAC has been shown to inhibit hydroxyl radical production in the presence of free iron or iron chelates. It should therefore be possible to use DETAPAC to probe for reactions (2) and (3) in some of the many systems in which a Haber-Weiss-type reaction has been postulated. Use of the chelator in this way has shown that an iron-catalysed Haber-Weiss reaction is not responsible for generation of 'OH radicals in the peroxidase/dihydroxyfumarate system.

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