Site-specific and bulk-phase generation of hydroxyl radicals in the presence of cupric ions and thiol compounds

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Addition of a thiol compound to a solution containing Cu^{2+} and H_2O_2 resulted in the generation of hydroxyl radicals ($\text{OH}^\bullet$). These radicals were able to oxidize salicylic acid and tryptamine in a reaction that was strongly inhibited by the $\text{OH}^\bullet$ -scavenger mannitol. Covalent coupling of the thiol compound to tryptamine did not significantly influence the degradation of the indole moiety subsequent to addition of H_2O_2 and Cu^{2+} . The inhibiting effect of mannitol, however, was strongly reduced, indicating that the scavenger could not interfere with site-specific reactions of $\text{OH}^\bullet$.

INTRODUCTION

The role of activated oxygen species as aetiological factor in the development of ostensibly very different pathological processes has been stressed increasingly in recent literature (Halliwell & Gutteridge, 1984; Slater, 1984; Cerutti, 1985). Frequently hydroxyl radicals ($\text{OH}^\bullet$), generated by an iron salt- or copper salt-catalysed Fenton-type reaction, are implicated as toxic species. This Fenton-type reaction requires the presence of both H_2O_2 and superoxide anions ($\text{O}_2^{\bullet-}$). Evidence for the generation of $\text{OH}^\bullet$ by this mechanism in the presence of iron salts or certain iron chelates is overwhelming. With Cu^{2+} as catalyst, however, the situation is more complicated. In biological systems Cu^{2+} binds to proteins, amino acids and other ligands, preventing the formation of $\text{OH}^\bullet$ in free solution. However, experimental results indicate that $\text{OH}^\bullet$, still generated via protein-bound Cu^{2+} , can induce site-specific protein damage at or near the binding site (Gutteridge & Wilkins, 1983; Samuni *et al.*, 1984).

It has been shown that generation of $\text{OH}^\bullet$ also occurs in a system containing Fe^{2+} (or Fe^{3+} -EDTA), H_2O_2 and a thiol compound. It is assumed that in this system $\text{O}_2^{\bullet-}$, necessary for the Fenton reaction, is generated by oxidation of thiols (Rowley & Halliwell, 1982). In the present paper it is shown that similar results are obtained with Cu^{2+} instead of Fe^{2+} . Depending on experimental conditions the $\text{OH}^\bullet$ generated in this system can participate in bulk-phase or site-specific reactions. The experimental results provide direct evidence for the inability of the $\text{OH}^\bullet$ -scavenger mannitol to interfere with site-specific reactions of $\text{OH}^\bullet$.

METHODS

Reaction mixtures (2 ml) contained (final concentrations) 0.5 mM- H_2O_2 , 0.1 mM of a thiol compound and 0.1 mM of either cupric acetate or Fe^{3+} -EDTA in 50 mM-sodium phosphate buffer, pH 7.4. All solutions were prepared in Chelex-resin-treated distilled water. As $\text{OH}^\bullet$ -sensitive substrate either salicylic acid (2 mM) or tryptamine (0.1 mM) was present. In some experiments tryptamine plus the thiol compound were replaced by 0.1 mM-*N*-acetylhomocysteinytryptamine, obtained by covalent coupling of tryptamine and *N*-acetylhomocysteine thiolactone by the method described by Cuatrecasas (1970). *N*-Acetylhomocysteinyglycine was

prepared by covalent coupling of glycine and *N*-acetylhomocysteine thiolactone by the same method (Cuatrecasas, 1970).

Reactions proceeded at room temperature. Hydroxylation of salicylic acid was assayed by the method of Richmond *et al.* (1981). Degradation of the indole moiety of tryptamine was measured by the method of Cowgill (1963). Fluorescence was monitored on an Aminco SPF500 spectrofluorimeter.

RESULTS

Hydroxylation of salicylic acid in the presence of Fe^{3+} -EDTA, H_2O_2 and *N*-acetylhomocysteinyglycine is shown in Fig. 1. The reaction is strongly inhibited by

Fig. 1. Generation of $\text{OH}^\bullet$, as reflected by hydroxylation of salicylate, in a reaction mixture containing *N*-acetylhomocysteinyglycine, H_2O_2 and either Fe^{3+} -EDTA or Cu^{2+}

For experimental details see the text. ●, Fe^{3+} -EDTA; ▲, Fe^{3+} -EDTA + 10 mM-mannitol; ○, Cu^{2+} ; △, Cu^{2+} plus 10 mM-mannitol.

Table 1. Production of OH[•] as reflected by hydroxylation of salicylic acid

The complete reaction mixture (2 ml) contained (final concentrations) 2 mM-salicylic acid, 0.1 mM-*N*-acetylhomocysteinylglycine (RSH), 0.1 mM-Cu²⁺ and 0.5 mM-H₂O₂. The final concentration of additions were: Fe³⁺-EDTA, 0.1 mM; mannitol, 10 mM; superoxide dismutase (SOD), 10³ units (μmol/min)/ml.

Reaction mixture	Additions	OH [•] production (nmol of salicylate hydroxylated/h)
Complete	None	37
- RSH	None	4
- H ₂ O ₂	None	3
- Cu ²⁺	None	0
Complete	Mannitol	10
Complete	SOD	29
- Cu ²⁺	Fe ³⁺ -EDTA	34
- Cu ²⁺	Fe ³⁺ -EDTA + mannitol	9
- Cu ²⁺	Fe ³⁺ -EDTA + SOD	6

mannitol, confirming the involvement of OH[•], in accordance with previous observations made by Rowley & Halliwell (1982). Hydroxylation of salicylic acid also occurred when the Fe³⁺-EDTA complex was replaced by Cu²⁺ (Fig. 1).

Replacement of *N*-acetylhomocysteinylglycine by reduced glutathione or dithiothreitol had no significant influence on the velocity of OH[•] formation (results not shown). Hydroxylation of salicylic acid was strongly inhibited by omission of either the thiol compound or H₂O₂, whereas in the absence of a metal ion catalyst no reaction took place (Table 1). Mannitol inhibited Cu²⁺-catalysed hydroxylation of salicylic acid with the same efficiency as it inhibited Fe³⁺-EDTA-catalysed hydroxylation. Superoxide dismutase, on the other hand, strongly diminished Fe³⁺-EDTA-mediated formation of OH[•], whereas Cu²⁺-induced generation of OH[•] was much less sensitive (Table 1).

McCord & Day (1978) described destruction of the indole moiety of tryptophan by OH[•] generated by an Fe³⁺-EDTA/xanthine/xanthine oxidase system. As expected, a similar degradation of the indole moiety occurred in a reaction mixture containing tryptamine, *N*-acetylhomocysteinylglycine, Fe³⁺-EDTA and H₂O₂ (Fig. 2). When tryptamine plus *N*-acetylhomocysteinylglycine were replaced by *N*-acetylhomocysteinyltryptamine the velocity of degradation of the indole moiety was slightly increased. In both cases destruction of the indole moiety was strongly inhibited by mannitol (Fig. 2). When Fe³⁺-EDTA was replaced by Cu²⁺ degradation of the indole moiety proceeded with a slightly higher velocity. With tryptamine plus *N*-acetylhomocysteinylglycine the reaction was again strongly inhibited by mannitol. When tryptamine plus *N*-acetylhomocysteinylglycine were replaced by *N*-acetylhomocysteinyltryptamine, however, the inhibitory effect of mannitol was much smaller (Fig. 3).

DISCUSSION

In a reaction mixture containing Cu²⁺ and H₂O₂ a slow production of OH[•] takes place (Table 1). According to

Fig. 2. Degradation of the indole moiety of tryptamine in the presence of Fe³⁺-EDTA and H₂O₂

For experimental details see the text. Fluorescence is expressed in relative intensity. Curve A, tryptamine plus *N*-acetylhomocysteinylglycine; curve B, *N*-acetylhomocysteinyltryptamine; curve C, as curve A, 10 mM-mannitol present; curve D, as curve B, 10 mM-mannitol present.

Fig. 3. Influence of mannitol on OH[•]-induced degradation of indole moiety in the presence of Cu²⁺ and H₂O₂

For experimental details see the text. ●, Tryptamine plus *N*-acetylhomocysteinylglycine; ○, *N*-acetylhomocysteinyltryptamine.

Gutteridge & Wilkins (1983), this slow production of OH[•] under these conditions can probably be accounted for by the reactions:

A similar slow generation of OH[•] occurs in the presence of thiols and Cu²⁺ (Table 1). This can be explained by

the fact that during oxidation of thiols O₂^{•-} is produced (Rowley & Halliwell, 1982; Misra, 1974; Saez *et al.*, 1982; Harman *et al.*, 1984). Dismutation of superoxide yields H₂O₂ and subsequently a Fenton-type reaction produces OH[•]:

A much faster production of OH[•] occurs in a reaction mixture containing a thiol, Fe³⁺-EDTA and H₂O₂ (Fig. 1 and Table 1), as described previously by Rowley & Halliwell (1982). According to these authors OH[•] is formed by a Fenton-type reaction, with O₂^{•-} derived from oxidation of thiol groups, as discussed above. In accordance with this, the reaction is inhibited by superoxide dismutase (Table 1 and Rowley & Halliwell, 1982).

As shown in the Results section Cu²⁺ catalyses the reaction even more effectively than does Fe³⁺-EDTA. A striking difference between the Fe³⁺-EDTA- and Cu²⁺-catalysed reactions is the much lower sensitivity to superoxide dismutase of the latter (Table 1). This may be attributed to the well-known non-specific binding tendency of Cu²⁺ to amino acids and other ligands (Gutteridge & Wilkins, 1983). Such a non-specific binding may cause a close proximity between Cu²⁺ and the superoxide-producing thiol groups. In this situation the reduction of Cu²⁺ by O₂^{•-} would be a site-specific reaction, explaining the inability of superoxide dismutase to interfere effectively with the reaction. Alternatively it is possible that O₂^{•-} is not involved in the thiol-mediated reduction of Cu²⁺. According to Misra (1974), Cu²⁺ can initiate thiol oxidation by the reaction:

Both possible explanations imply a close proximity between Cu²⁺ and the thiol groups.

As described by McCord & Day (1978), OH[•] can cause degradation of indole groups. This was confirmed by the results shown in Fig. 2. In a reaction mixture containing tryptamine, *N*-acetylhomocysteinylglycine, H₂O₂ and either Fe³⁺-EDTA or Cu²⁺ the destruction of the indole moiety was strongly inhibited by the OH[•]-scavenger mannitol, as expected (Figs. 2 and 3).

Previous experimental results suggest that the protective effect of OH[•] scavengers is substantially lessened when OH[•] is generated in close proximity to its target in a site-specific reaction (Gutteridge & Wilkins, 1983; Samuni *et al.*, 1984). The results shown in Fig. 3 appear to be a direct demonstration of this phenomenon. When tryptamine plus *N*-acetylhomocysteinylglycine were replaced by *N*-acetylhomocysteinyltryptamine the velocity of degradation of the indole moiety after addition of Cu²⁺ and H₂O₂ was slightly increased. The inhibition by mannitol, on the other hand, was much smaller. As discussed above, the reacting Cu²⁺ is presumably in the direct vicinity of the thiol group. Therefore with *N*-acetylhomocysteinyltryptamine generation of OH[•] in accordance with eqn. (5) will occur in close proximity to its target, in view of the presence of the indole moiety and thiol group in the same molecule. Fig. 3 clearly demonstrates the lack of protection by the OH[•]-scavenger mannitol in this site-specific reaction.

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