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Formation of Superoxide Radical Anion in the Horseradish Peroxidase-catalysed Oxidation of Three Aromatic Tertiary Amines with Hydrogen Peroxide

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The horseradish peroxidase-catalysed oxidation of aromatic tertiary amines with hydrogen peroxide to give a secondary amine and an aldehyde occurs through the co-occurrence of the usual reaction with H_2O_2 and a free radical chain reaction which depends upon oxygen concentration. The intervention of superoxide radical anion is demonstrated by the reduction of cytochrome c^{III} and the dismutation catalysed by superoxide dismutase.

HORSERADISH peroxidase (HRP, EC 1.11.1.7, donor H_2O_2 oxido-reductase) catalyses the oxidation of a great number of compounds by hydrogen peroxide. The HRP-catalysed oxidation of *NN*-dialkylanilines with hydrogen peroxide to yield *N*-alkylanilines and an aldehyde [reaction (1)] was shown by us to be accompanied by an oxidative chain process where oxygen is the oxidising agent [reaction (2)].[1] By analogy with the

$$XC_6H_4NMe_2 + H_2O_2 \longrightarrow XC_6H_4NHMe + CH_2O + H_2O \quad (1)$$

$$XC_6H_4NMe_2 + O_2 \xrightarrow{2H^+} XC_6H_4NHMe + CH_2O + H_2O \quad (2)$$

mechanistic pattern for the oxidation of tertiary amines [2,3] equations (3)—(5) were proposed to occur in the reaction with H_2O_2 given that the oxidising agent is

$$XC_6H_4NMe_2 \xrightarrow{ox} XC_6H_4\overset{+\cdot}{N}Me_2 \quad (3)$$

$$XC_6H_4\overset{+\cdot}{N}Me_2 \longrightarrow XC_6H_4NMeCH_2^{\bullet} + H^+ \quad (4)$$

$$XC_6H_4NMeCH_2^{\bullet} \xrightarrow{ox} XC_6H_4NMeCH_2^+ \longrightarrow XC_6H_4NHMe + CH_2O \quad (5)$$

either the peroxidase compound E_1 or E_2, generated by the interaction of HRP with hydrogen peroxide followed by the reaction with an organic substrate AH_2 as shown in equations (6)—(8).[4,5]

$$HRP + H_2O_2 \longrightarrow E_1 \quad (6)$$

$$E_1 + AH_2 \longrightarrow E_2 + AH^{\bullet} \quad (7)$$

$$E_2 + AH_2 \longrightarrow HRP + AH^{\bullet} \quad (8)$$

A species such as the α-alkylarylamino radical $XC_6H_4NMeCH_2^{\bullet}$ could be responsible for the reaction with oxygen. Two mechanisms could account for the activation of molecular oxygen. (a) An intermediate radical species undergoes electron transfer with oxygen to give a carbonium ion and $O_2^{\bullet -}$, as suggested for some oxidative processes due to peroxidase [6,7] [equation (9)]. The superoxide radical anions thus formed could either undergo a rapid dismutation to give a molecule of oxygen and a molecule of hydrogen peroxide [equation (10)] or

$$XC_6H_4NMeCH_2^{\bullet} + O_2 \longrightarrow XC_6H_4NMeCH_2^+ + O_2^{\bullet -} \quad (9)$$

$$2\,O_2^{\bullet -} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \quad (10)$$

$$XC_6H_4NMeCH_2^{\bullet} + O_2^{\bullet -} + 2\,H^+ \longrightarrow XC_6H_4NMeCH_2 + H_2O_2 \quad (11)$$

undergo oxidation to give a carbonium ion and hydrogen peroxide [equation (11)]. In both cases hydrogen peroxide would be recycled. (b) The intermediate (pH 7.4, k 2×10^5 l mol^{-1} s^{-1}; catalysed by superoxide dismutase k 2×10^9 l mol^{-1} s^{-1}) [8] radical species reacts with oxygen forming a covalent bond. The hydroperoxyl radical thus formed behaves as hydrogen peroxide by oxidising HRP [equations (12) and (13)].

$$XC_6H_4NMeCH_2 + O_2 \longrightarrow XC_6H_4NMeCH_2\text{-}O\text{-}O^{\bullet} \quad (12)$$

$$XC_6H_4NMeCH_2\text{-}O\text{-}O^{\bullet} + HRP \longrightarrow XC_6H_4NHMe + CH_2O + E_2 \quad (13)$$

The consumption of oxygen has been reported in the HRP-catalysed oxygenation of 2′,4,4′-trihydroxychalcone,[9] indol-3-ylacetic acid, dihydroxyfumarate, and reduced nicotine adenine dinucleotide.[10] In the first case a chalcone cyclic peroxide was isolated and characterised. Path (b) has been also proposed for the oxidation of indol-3-ylacetic acid to give mainly 3-methylene-2-oxoindole. A study of kinetic inhibition by superoxide dismutase supported this mechanism. The same method led to the suggestion that the oxidation of dihydroxyfumarate and reduced nicotine adenine dinucleotide follows path (a).

Here we report some evidence for the formation of superoxide radical anion [path (a)] in the reaction of 4-fluoro-, 4-chloro-, and 3-chloro-dimethylaniline (DMAs) with hydrogen peroxide in the presence of HRP and oxygen. These amines belong to a larger series of substrates tested.[1] For these compounds the requirements of absence of by-products and accuracy of spectroscopic measurements were met.

RESULTS AND DISCUSSION

The rapid reduction of cytochrome c^{III} (cyt c^{III}) by $O_2^{\cdot-}$ [equation (14)] and the fact that this reaction is replaced by the dismutation of $O_2^{\cdot-}$ in a very fast process in the presence of the enzyme superoxide dismutase [equation (10)], have been widely used as a diagnostic tool for the presence of $O_2^{\cdot-}$.[11,12]

$$\text{cyt } c^{III} + O_2^{\cdot-} \longrightarrow \text{cyt } c^{II} + O_2 \qquad (14)$$
$$(k\ 10^6\ \text{l mol}^{-1}\ \text{s}^{-1})\ [12]$$

When the HRP-catalysed oxidation of 4-F-DMA with hydrogen peroxide was performed in the presence of cyt c^{III}, the appearance at 550 nm of the absorbance of cyt c^{II} formed *via* reaction (14) was observed. This was a qualitative measure of the presence of $O_2^{\cdot-}$ because some of the cyt c^{II} formed was oxidised by hydrogen peroxide in the presence of HRP [equation (15)] at a rate slower than the reduction by $O_2^{\cdot-}$ and similar to that of the HRP-catalysed oxidation of 4-F-DMA to 4-fluoro-*N*-methylaniline and formaldehyde (*k ca.* 10 l mol^{-1} s^{-1}).

$$2\ \text{cyt } c^{II} + H_2O_2 + 2\ H^+ \xrightarrow{\text{HRP}} 2\ \text{cyt } c^{III} + 2\ H_2O \qquad (15)$$
$$(k\ 1\text{—}100\ \text{l mol}^{-1}\ \text{s}^{-1})\ [13]$$

A quantitative approach to the problem of the presence of $O_2^{\cdot-}$ was suggested from the observation that at 325 nm, the isosbestic point for the couple cyt c^{III}–cyt c^{II}, the oxidation of a substituted *NN*-dimethylaniline to the corresponding *N*-methylaniline produces a variation of optical density which can be correlated to the extent of the reaction. The degree of conversion of two substituted *NN*-dimethylanilines in the absence (α) and in the presence of cty c^{III} (α') has been measured. The reaction was performed at 25 °C with [amine] 5×10^{-4}M, [EDTA] 10^{-4}M, [HRP] 50 μg l^{-1}, and phosphate buffer at pH 7.4. The value $[1-(\alpha'/\alpha)]\ 100 = R$ is a measure of the percentage lowering of the degree of transformation by subtracting $O_2^{\cdot-}$. The values of R for different concentrations of cyt c^{III} and hydrogen peroxide are reported in Table 1 (mean of three values).

These values indicate that the addition of cyt c^{III} leads to a decrease of the degree of conversion of the amine due to competition between the oxidation of the α-alkylarylamino radical by $O_2^{\cdot-}$ and the consumption of $O_2^{\cdot-}$ *via* reaction with cyt c^{III} (reaction (14)], taking into account that the amount of cyt c^{III} in solution is also influenced by the HRP-catalysed reoxidation of cyt c^{II} [reaction (15)]. This competition seems not to hold for very low concentrations of cyt c^{III}. A further proof of the formation of $O_2^{\cdot-}$ in this reaction is obtained by the fact that addition of superoxide dismutase (SOD) to the reaction performed in the presence of cyt c^{III} leads to a smaller decrease of the degree of conversion. Table 2 shows the values of R (mean of three values) obtained for different concentrations of SOD in runs at 25 °C with [4-F-DMA] 5×10^{-4}M, [H_2O_2] 1.44×10^{-4}M, [cyt c^{III}] 1.25×10^{-6}M, [HRP] 50 μg l^{-1}, [EDTA] 10^{-4}M, and phosphate buffer at pH 7.4. These data suggest that the catalytic enhancement of the dismutation rate of reaction (10), which produces hydrogen peroxide and carries on the oxidation of the amine, overcomes the consumption of $O_2^{\cdot-}$ by cyt c^{III}. Moreover, strong deactivation of SOD due to hydrogen peroxide seems to be present.[14]

Further evidence of the presence of $O_2^{\cdot-}$ is obtained from the results shown in Table 3, indicating the variation of R with HRP and H_2O_2 concentrations. These data show that the degree of conversion of the amines is correlated almost linearly with the concentration of HRP. This accords well with the results described by Fridovich on the reduction of cyt c^{III} by the $O_2^{\cdot-}$ generating system xanthine–xanthine oxidase–oxygen. It was demonstrated that at saturating concentrations of cyt c^{III} the initial rates of reduction should be directly proportional to the concentration of xanthine oxidase.[15] In our case such a dependence could be related to the fact that the concentration of the enzyme allows the presence of a steady state level of organic radical species. The different amounts of HRP used for different substrates derive from the fact that the initial rates follow the order 4-F-DMA > 4-Cl-DMA > 3-Cl-DMA.

TABLE 1

	$10^4[H_2O_2]$/M			
	0.48	0.96	1.44	
10^6[cyt c^{III}]/M	*R*	*R*	*R*	Substrate
2	28	36	29	
1.25	30	33	30	
1	9	14	10	4-F-DMA
0.833	0	0	0	
0.416	0	0	0	
2	25	25	27	4-Cl-DMA
1.5	16	17	16	
1	0	0	0	

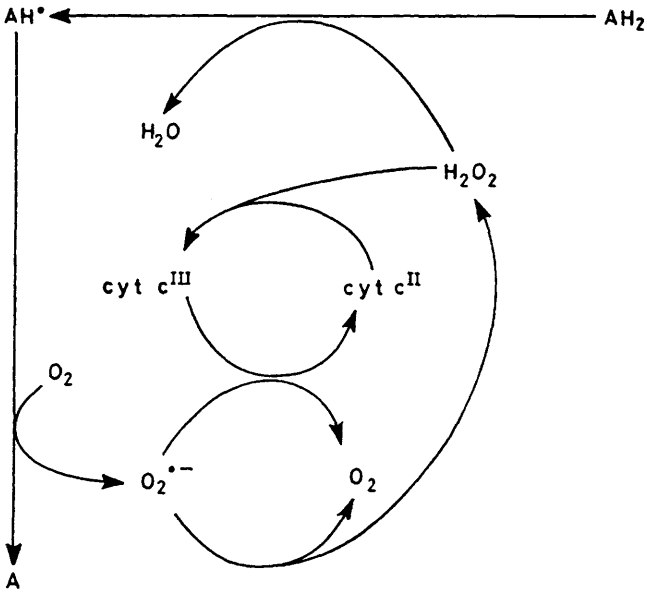
TABLE 2

[SOD]/μg ml^{-1}	0	0.2	1	5
R	33	30	26	10

TABLE 3

	$10^4[H_2O_2]$/M			
	0.48	0.96	1.44	
[HRP]/mg l^{-1}	*R*	*R*	*R*	Substrate
0.5	12	10		4-F-DMA
1	15	15		
1.5	18	15		
6.25	25	26		
25	13	12	12	4-Cl-DMA
50	25	25	27	
75	47	42	43	
50		0	0	3-Cl-DMA
150		4	3	

The results allow the following conclusions to be drawn. During the HRP-catalysed reaction of amines with hydrogen peroxide the superoxide radical anion is formed and its steady state concentration depends upon the rate of oxidation of the amine by hydrogen peroxide. Superoxide radical anions can either dismutate or reduce cyt c^{III}. The latter reaction prevents the recycling of hydrogen peroxide and, therefore, lowers the extent of the overall oxidation of the amine. The amount of $O_2^{\bullet -}$ which follows this path is kinetically correlated with the concentration of cyt c^{III}.



SCHEME

Moreover, cyt c^{II} thus formed is enzymatically oxidised back to cyt c^{III} by one equivalent of hydrogen peroxide. In summary, each superoxide radical anion shifted to the cyt c^{III} reductive process subtracts two oxidative equivalents from the oxidation of the amine. These observations are summarised in the Scheme. Finally, the specificity of the action of superoxide dismutase seems to make the intermediacy of hydroperoxyl radicals formed *via* path (b) less probable.

EXPERIMENTAL

Materials.—*NN*-Dialkylanilines were prepared by known methods. A standard solution of HRP (Boehringer grade II; 1% in water) was prepared, kept in a refrigerator, and used within a few days. Cytochrome c^{III} was purchased from Boehringer. Hydrogen peroxide was a Baker analysed reagent and was tested iodometrically. Reactions were performed in a buffer solution made up with NaH_2PO_4,-H_2O (15.4 g l^{-1}) $NaHPO_4$, $12H_2O$ (2.7 g l^{-1}), and EDTA disodium salt (37 mg l^{-1}). Salts were purchased from Carlo Erba. Lyophilised SOD was dissolved in the reaction medium just before use. Absorbances were measured using a Hitachi–Perkin-Elmer 124 spectrophotometer.

Stoicheiometric Experiments.—Each determination of the value of R was performed as follows. (a) Two 50 mm cells were filled with the same amount (14 ml) of a solution containing the required amount of HRP and amine, and placed in the spectrometer as both sample and reference. The reaction was started by adding the required amount of a solution of hydrogen peroxide to the sample cell with a microsyringe. Dilution of the stock solution of hydrogen peroxide was such as to allow additions ranging from 10 to 90 μl. α was recorded as the variation of optical density at 325 nm. (b) The sample cell was filled with equal amounts (7 ml) of a solution containing HRP and amine and a solution of cyt c^{III}. In the reference cell the solution (7 ml) of HRP and amine was diluted with an equal amount of buffer. The reactions were started by adding equal amounts of hydrogen peroxide to both cells, α' was recorded as the variation of optical density at 325 nm. The experiments with SOD were performed by adding the required amount of the enzyme to the sample cell containing amine, HRP, and cyt c^{III}.

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