IS SINGLET OXYGEN A SUBSTRATE FOR SUPEROXIDE DISMUTASE? NO

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1. Introduction

Since the discovery of the enzymatic function of erythrocuprein as a dismutase of superoxide radicals by McCord and Fridovich [1,2] a large number of publications have described the biological production of O_2^-, the characterisation and properties of various superoxide dismutases (metalloproteins containing copper, manganese or iron), and the protective role of such enzymes [3–5]. In addition, the catalytic effect of superoxide dismutases in the destruction of O_2^- has been followed by direct observation of the substrate using advanced physical techniques [6]. In a recent communication Agro et al. [7] claim that 'erythrocuprein has a quenching effect on luminescence phenomena which are reasonably ascribed to singlet oxygen produced even in the absence of superoxide intermediates'. This conclusion was based on studies of the lipoxidase–linoleate system. The present note indicates that the conclusion is unjustified.

2. Materials and methods

Bovine erythrocuprein was prepared according to Stansell and Deutsch [8] with minor modifications. The enzyme was completely free of peroxidase, catalase or oxidase activity. Lipoxidase was a commercial preparation (Sigma) which was further purified by passage through a column of Chelex. No peroxidase or catalase activity could be detected in the final preparation. Light emission was measured in an apparatus which has been previously described [9]. Superoxide dismutase units are those defined elsewhere [5]. Pure bovine erythrocuprein has a specific activity of 140 000 of these units per mg. The purification of *Photobacterium leiognathi* superoxide dismutase (a ferroprotein) and the characteristics of this enzyme are described elsewhere [10]. Again no peroxidase, oxidase or catalase activity could be detected. Denatured enzymes were prepared by heating dilute solutions at 100°C for 10 min. Both erythrocuprein and bacterial superoxide dismutase were used in duplicate experiments.

3. Results

No emission of light could be observed when lipoxidase (up to 3000 units/ml) was incubated with linoleic acid under the following conditions, contrary to the observations of Agro et al. [7]. Appropriate quantities of lipoxidase (specific activity 250 000 units/mg) were placed in the cuve followed by 1 ml of 0.2 M borate buffer pH 9.0. The reaction was initiated by injection of 2 ml of a solution of linoleic acid prepared by diluting 10 μl of linoleic acid in 10 μl of ethanol with 10 ml of water, followed by addition of 50 ml of borate buffer. In this system 1000 units/ml cause an increase in absorption at 234 nm of 1 OD per min at 25°C. To verify this negative result, an Intertechnique scintillation counter was also used; the result was equally negative. If light is emitted during the action of lipoxidase on linoleic acid it is less than 100 quanta per sec per ml at 480 nm. Since chemiluminescence of luminol can easily give rise to 10^{14} quanta/sec/ml it may be considered that light emission from lipoxidase/linoleic acid is less than 10^{-12} that of a typical chemiluminescent reaction. The observation by Agro et al. [7] of 'a strong chemiluminescence' is thus the result of other factors. Indeed the increase in light emission in the presence of hydrogen peroxide which is further increased in the presence of erythrocuprein observed

by these authors is probably indicative of general contamination by peroxidases, rather than suggestive that linoleate peroxides interact with erythrocuprein.

3.1. *Light emission of luminol and Pholad luciferin*

Both luminol and Pholad luciferin can act as efficient detectors of superoxide ions or other free radicals, the luciferin being some 10–50 times more efficient than luminol [11]. Since direct evidence of production of superoxide radicals by the system lipoxidase $-O_2-$ linoleic acid could not be obtained, the enzymic reaction was performed in presence of luminol (10^{-4} M) and Pholad luciferin (10^{-9} M). Oxidation of luciferin with concomitant light emission did indeed occur, but was completely insensitive to superoxide dismutase (fig. 1) and hence was due to the action of free radicals other than O_2^- produced in the lipoxidase system. We have previously shown that chemiluminescence of luciferin induced by O_2^- is indeed sensitive to superoxide dismutase [11]. Similar light emission was observed if the luciferin was injected 6 min after initiation of the lipoxidase–linoleic reaction. A very slight stimulation of Imax was observed but no significant indication of accumulation of radical intermediates could be observed, and in fact, as may be expected, Imax is a function of the quantity of lipoxidase used and is six times greater if 3000 units/ml of lipoxidase is used rather than 500 (fig. 1). Presence of 2×10^{-4} M Fremy's salt [12,13] well known as a free radical trap caused 100% inhibition of light emission due to Pholad luciferin.

The results with luminol were somewhat different. A lower level of light emission is obtained (fig. 2) and this is inhibited by superoxide dismutase (75% with 25 units SOD/ml and 83% with 50 units SOD/ml) but not by heat denatured SOD. Again, 2×10^{-4} M Fremy's salt causes 100% inhibition.

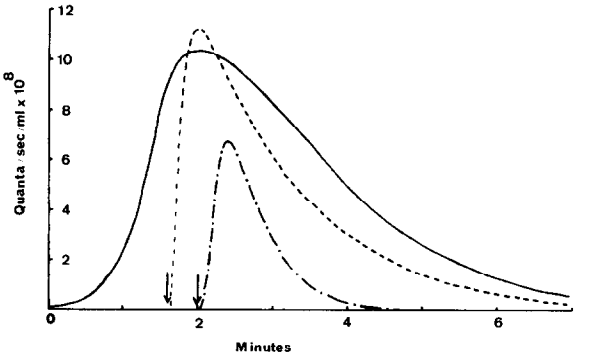

Fig. 1. Light emission of *Pholad* luciferin (10^{-9} M) in presence of lipoxidase (500 units/ml) – linoleic acid. Reaction is initiated by injection of the solution of linoleic acid. The broken line (—) shows light emission if the luciferin is injected 6 min after initiation of the reaction lipoxidase–linoleic acid. Light emission with 3000 units lipoxidase/ml is also shown (–·–·–) with a 10-fold reduced light scale. Conditions as in text. Arrows indicate initiation by injection of linoleate.

It may be noted that at the concentrations cited, luciferin, luminol, Fremy's salt or superoxide dismutase (up to 350 units/ml) had absolutely no effect on the kinetics of oxidation of linoleic acid by lipoxidase, and that neither luciferin nor luminol are oxidised by lipoxidase alone.

3.2. *Photoreduction of Methylene Blue for production of superoxide radicals*

Production of excited singlet oxygen species via photosensitizers such as Methylene Blue is often cited as the mechanism of certain oxidations. Methylene Blue

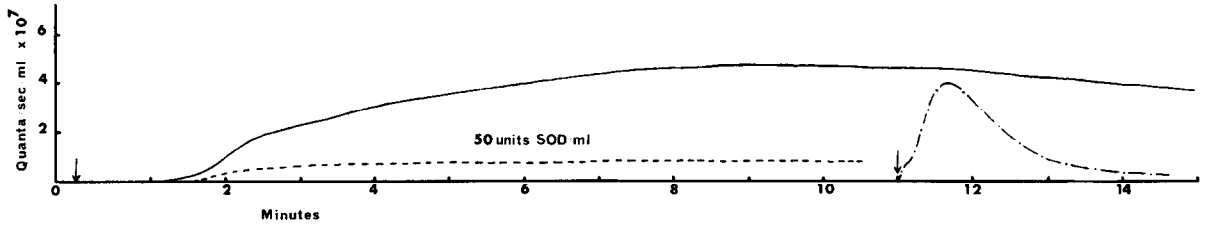

Fig. 2. Light emission of luminol (10^{-4} M) in presence of lipoxidase (500 units/ml)-linoleic acid in absence (——) and in presence (– – –) of 50 units/ml of superoxide dismutase. Light emission with 3000 units lipoxidase/ml is also shown (–·–·–·–) with a 10-fold reduced light scale. Arrows indicate initiation by injection of linoleate.

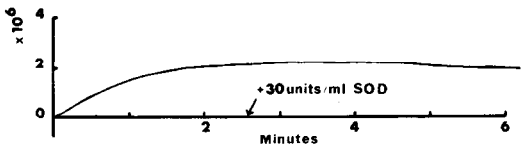

Fig. 3. Oxidation of luminol with reduced Methylene Blue/O_2. A solution of 10^{-4} M methylene blue in 10^{-4} M EDTA, 5×10^{-3} M phosphate pH 7.8 was photoreduced anaerobically then 1 ml of this solution was injected into 10^{-4} luminol in 5×10^{-2} M ammonium acetate pH 9.3.

(10^{-4} M) in 5×10^{-3} M phosphate pH 7.8 containing 10^{-4} M EDTA was photoreduced after degassing with argon. The anaerobic solution (1 ml) was then injected into a cuvette containing 10^{-4} M luminol in 5×10^{-2} M ammonium acetate pH 9.3. Light emission was observed (fig. 3) but was completely inhibited with 30 units SOD/ml whereas the same amount of heat denatured enzyme was completely without effect. The major cause of aerobic photosensitized oxidations using methylene blue (in presence of a donor such as EDTA) is thus formation of superoxide radicals rather than excited singlet oxygen.

4. Discussion

No evidence whatsoever has been presented by Agro et al. [7] 'that erythrocuprein can quench singlet oxygen even if superoxide intermediates are not present'. It would thus appear reasonable to consider that the substrate of superoxide dismutase is indeed the superoxide radical and not an entity with a completely different electronic structure.

With respect to the bleaching of cytochrome *c* by lipoxidase–linoleate and the 'inhibition' of this bleaching by enormous quantities of erythrocuprein (5×10^{-7} M corresponds to some 2500 units/ml and causes only 30% inhibition) reported by Agro et al. [7], in the absence of a suitable control with denatured erythrocuprein it can only be assumed that proteins other than cytochrome *c* react readily with free radical intermediates. In this respect it may be noted that denatured superoxide dismutase is equally as effective as the native enzyme in inhibiting the oxidation of Pholad luciferin by nitroso-diethylamine.

The mechanism of lipoxidase as generally accepted involves formation of a radical of linoleic acid by removal of hydrogen, followed by reactions of this radical with oxygen and return of the hydrogen to give a hydroperoxide [14,15], that is, activation of linoleic acid and not oxygen (as is the case with a number of dioxygenases) occurs. That these intermediates are enzyme bound (and hence protected) is shown by the fact that Fremy's salt does not inhibit the reaction. Nevertheless some leakage can occur (a function of for example the K_m of linoleate radical for the enzyme) leading to an exchange of the type LH_2 + linoleate radical (or linoleate peroxy radical) → $LH^{\cdot}$ + linoleate or linoleate hydroperoxide (where LH_2 represents luciferin). The reactions leading to light emission are then

$$LH^{\cdot} + O_2 \rightarrow LHOO^{\cdot}; \quad LHOO^{\cdot} + LH_2 \rightarrow LHOOH + LH^{\cdot};$$

$$LHOOH \rightarrow \text{oxyluciferin} + h\nu$$

It is clear that in this case, superoxide dismutase cannot inhibit the reaction, whereas Fremy's salt will immediately block any free linoleate radicals and hence ultimate light emission. Oxidation of luciferin thus resembles oxidation of cholesterol by the system lipoxidase–linoleate [16].

We now consider the case of luminol. Whereas the luciferin was at 10^{-9} M we now have a 10^5 increase in concentration of the scavenger. In addition we must consider the relative redox potentials as well as the different bond energies to compare the reactivity with any possible intermediates in the lipoxidase–linoleate system. Again, Fremy's salt (a relatively stable free radical) must block any free radical initiation steps which could lead to light emission by oxidation of the luminol. A second possibility lies in the reaction of organic free radicals with oxygen, $LH^{\cdot} + O_2 \rightarrow L + HOO^{\cdot}$, that is, the luminol radical itself produced as described above, can give rise to superoxide radicals which then oxidise luminol and the reaction is at least partially inhibited by superoxide dismutase.

The photoreduction of Methylene Blue and its autoxidation with production of $O_2^{\cdot-}$ provides a satisfactory mechanism for a number of photosensitized oxidations.

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