ERYTHROCUPREIN AND SINGLET OXYGEN*

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1. Introduction

McCord and Fridovich [1, 2] have shown that erythrocuprein, the copper and zinc containing protein extracted from red blood cells, can enzymatically disproportionate superoxide radicals ($O_2^{\cdot -}$), an intermediate of the reduction of oxygen, the generation of which in some H_2O_2-producing biological oxidations has been demonstrated [3]. Khan has recently pointed out [4] that dismutation of $O_2^{\cdot -}$ gives rise to the production of the highly reactive singlet state of oxygen, which can be revealed by its own luminescence; indeed Arneson [5] observed that oxidation of xanthine by xanthine oxidase produced luminescence, which was abolished by the presence of erythrocuprein. On the basis of these data, the main purpose of the following paper is to show that erythrocuprein has a quenching effect on luminescence phenomena which are reasonably ascribed to singlet oxygen produced even in the absence of superoxide intermediates. Thus the physiological role of erythrocuprein could be to catalyze the singlet–triplet conversion of the oxygen states: in other words to afford an enzyme-regulated dismutation liberating in solution as a product the inert ground state triplet oxygen, rather than simply accelerating the dismutation reaction.

* Dedicated to Prof. Alexander Braunstein, in occasion of his 70th birthday.

2. Materials and methods

Erythrocuprein was purified from ox blood according to McCord and Fridovich [1]. Copper-free erythrocuprein was prepared as previously described [6]. Horse heart cytochrome *c* (Type III) and soy bean lipoxidase (Type I) were obtained from Sigma; milk xanthine oxidase was purchased from Boehringer. Luminescence was observed with a Nuclear Chicago Model 725 liquid scintillation counter. The settings used were those suggested by Vorhaben and Steele [7] or Arneson [5]. The production of superoxide radicals was measured spectrophotometrically by the method of cytochrome *c* reduction [1].

3. Results

Two different types of luminescent systems in which the participation of singlet oxygen produced by different mechanisms is likely to occur were investigated. They are the xanthine–xanthine oxidase system and the lipoxidase–linoleate system.

3.1. *The xanthine–xanthine oxidase system*

Extremely low concentrations of erythrocuprein were able to quench the luminescence produced by incubating xanthine and xanthine oxidase at any pH tested (fig. 1), as already observed by Arneson at pH 7.8 [5]. In the same conditions the reduction of cyto-

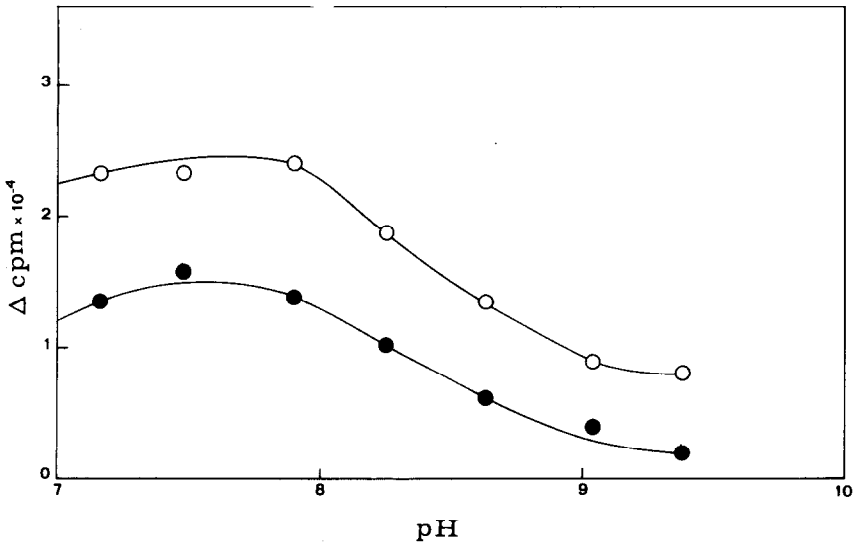

Fig. 1. Quenching of xanthine–xanthine oxidase luminescence by erythrocuprein as a function of pH. The incubation mixture contained in 10.6 ml of 0.05 M barbital buffer at various pH's, 7×10^{-8} M xanthine oxidase, 4×10^{-4} M xanthine and 4×10^{-4} M EDTA. (o—o—o): Without erythrocuprein; (•—•—•): with 6×10^{-8} M erythrocuprein. The reaction was started by addition of xanthine oxidase and performed at room temp.

chrome *c* was also inhibited. The addition of cytochrome *c* quenched the luminescence.

The action of erythrocuprein is specific: Cu-EDTA or azurin affect neither the luminescence nor the cytochrome *c* reduction even at much higher concentrations. Furthermore erythrocuprein was inactive when copper was removed: the addition of copper to copper-free erythroprein restored the activity.

The addition of H_2O_2 to the system produced a great increase of luminescence: in this case the addition of erythrocuprein was not only ineffective but also prolonged the luminescence decay. In the same conditions cytochrome *c* became fully oxidized.

3.2. *The lipoxidase–linoleate system*

When lipoxidase was incubated in the scintillation vials in the presence of linoleate, a strong chemilluminescence was produced which was quenched by minute amounts of erythrocuprein (10^{-7}–10^{-8} M). Control experiments showed that erythrocuprein had no inhibitory effect on lipoxidase. On the other hand, the addition of erythrocuprein after preincubations of 15–30 min produced an increase of luminescence. When cytochrome *c* was incubated in the presence of linoleate and lipoxidase, no reduction was observed; on standing, instead, a bleaching of the heme absorption occurred. The addition of erythrocuprein prevented this bleaching (fig. 2). In scintillation experiments the addition of cytochrome *c* at the concentrations used in the spectrophotometric experiments did not affect the luminescence.

4. Discussion

In the xanthine–xanthine oxidase system the inhibition by erythrocuprein of cytochrome *c* reduction is paralleled by an effective quenching of the luminescence produced by this system. In this case the latter effect may certainly be interpreted in terms of superoxide dismutase activity as indicated by the fact that cytochrome *c* added into the scintillation vials reduces the luminescence, probably by scavenging superoxide anions. However, erythrocuprein at concentrations as low as 10^{-8} M is able also to quench up to 80% the luminescence produced by incubating lipoxidase and linoleate. In this reaction neither reduction nor quenching effect of cytochrome *c* was observed. Thus, these types of evidence failed to show the possible production of $O_2^{\cdot-}$ by this system. Recently

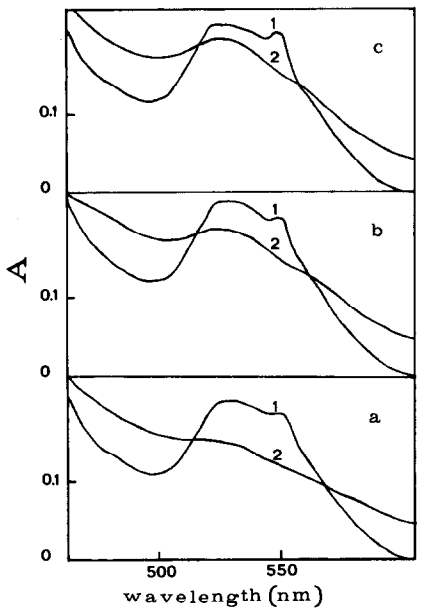

Fig. 2. Bleaching of cytochrome *c* absorption by the lipoxidase–linoleate system. The incubation mixture contained in 3 ml of 0.1 M Tris-HCl pH 9, 10^{-6} M lipoxidase, 6×10^{-3} M linoleate and 3×10^{-5} M cytochrome *c*. The spectra labeled 1 were recorded just before the addition of linoleate; those indicated by 2 were recorded after 45 min incubation at room temp in the presence of linoleate. a) Without erythrocuprein; b) 8×10^{-8} M erythrocuprein; c) 5×10^{-7} M erythrocuprein.

Chan [8] reported that lipoxidase in the presence of its substrate (linoleic acid) gives rise to a "singlet oxygen-like" intermediate. In our experiments the cleavage of the heme moiety in the presence of the lipoxidase–linoleate system may be attributed to singlet oxygen, which may be responsible also for luminescence.

It is important to note that whenever the luminescence is increased by addition of hydrogen peroxide, erythrocuprein is not only unable to act as a quencher, but even causes an increase of luminescence. This observation might explain the increase of luminescence produced by adding erythrocuprein to the lipoxidase–linoleate system later during incubation; in this case greater amounts of linoleate peroxides are formed by lipoxidase and they might interact with erythrocuprein in the same way as H_2O_2 does. It should pointed out that the electron spin resonance spectrum of erythrocuprein is modified after treatment with a slight excess of H_2O_2 (unpublished data from this laboratory), suggesting that this protein is sensitive to the action of proxides.

The possibility that erythrocuprein may act as a singlet oxygen quencher has already been proposed by Arneson [5]. However he assumed that the quenching was only secondary with respect to the dismutation of superoxide anions. The experiments reported above on the lipoxidase–linoleic acid suggest that erytrocuprein can quench singlet oxygen even if superoxide intermediates are not present. This quenching efficiency is specific and linked to copper. However, the catalytic efficiency of erythrocuprein appears to be greatly improved by its high affinity towards superoxide anions, which are the major source of singlet oxygen in aerobic organisms. This binding of superoxide anions is suggested by the inhibition of cytochrome *c* reduction and might be related to the special feature of the copper site of erythrocuprein [9]. The detailed mechanism of action of erythrocuprein as an enzyme will be the object of a further paper. At present it can be concluded that the effective function of erythrocuprein may be to protect bacterial and animal organisms against singlet oxygen which otherwise could "burn" anything around it. This suggestion fits fairly well with the ubiquity of erythrocuprein in animal cells, even those which do not seem to contain superoxide-producing enzymes as, for instance, red blood cells.

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