PRODUCTION OF SUPEROXIDE IONS BY PHOTOSENSITIZATION OF DYES

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Summary : The formation of superoxide anions by photosentization of fluorescein and other aromatic molecules has been observed using the enzyme lactoperoxidase as detector. Aerobic photosensitization caused the formation of Compound III with a rate of formation directly proportional to light intensity. Yields were such that photosensitization could be used as a superoxide anion generating process. Superoxide dismutase was also successfully used to demonstrate the specific involvement of superoxide ions in this photoprocess.

INTRODUCTION :

The univalent reduction of O_2 to O_2^- (superoxide anion) has been observed in a number of chemical and enzyme-catalyzed reactions. In most cases O_2^- was detected with the enzyme superoxide-dismutase, which catalyzes the dismutation of O_2^- into O_2 and H_2O_2 (1-8). A similar process was observed during the reoxidation of photoreduced flavin in air (9) and quite recently in photolyzed water (10).

In the present paper, we wish to report the direct demonstration that O_2^- is produced in substantial yields during the photosensitization of dyes or aromatic compounds in the presence of molecular oxygen. Kinetic and chemical evidence for the formation of molecular complexes of ion pair type such as $D^+ \ldots O_2^-$ has been presented (11) and we tried to identify the free oxidizing species O_2^- by its reaction with lactoperoxidase (Fe_p^{3+} - H_2O). It is known that such an enzyme reacts with O_2^- to give an intermediate compound - compound III - which is stable under suitable conditions (12).

We also used horseradish peroxidase to test for the formation of hydrogen peroxide in the system by working at cryogenic temperatures in a fluid mixture of ethylene glycol and buffer. Under these conditions compounds I and II, formed by reaction with hydrogen peroxide, are stable. We confir-

med the production of O_2^- by the inhibitory effect of superoxide-dismutase on the formation of compound III.

MATERIALS AND METHODS :

The dye used as a photosensitizer was fluorescein (from Merck) in aqueous buffered solutions (0.1M cacodylate, pH 6.2) at room temperature, and in a mixture of ethylene glycol and water (v/v = 1:1) at -45°. Ethylene glycol was from Carlo Erba. Lactoperoxidase used to trap O_2^- ions, and catalase used to test for the formation of H_2O_2 were from Sigma, and superoxide dismutase from Miles. Nicotinamide adenine dinucleotide phosphate (NADPH) used as a photosensitizer was from Sigma.

Photosensitization was accomplished by illuminating solutions in quartz cuvettes with a light-path lenght of 10 mm, in an appropriate cryostat (with a 450 W xenon vapor lamp (from Osram). Filters Corning 4308 and 9863 were respectively used to irradiate the fluorescein and NADPH. Neutral density filters were used to perform irradiations at different intensities. Absorption spectra and absorbance changes were measured in a Cary 15 spectrophotometer. Experiments were performed in a range from 20° to -45°. A temperature control device and a suitable cryostat, described elsewhere (13), permitted one to maintain the samples at any desired temperature in this range of temperature.

The behaviours of the enzymes, lactoperoxidase and catalase, in ethylene-glycol-water mixtures at sub-zero temperatures were checked by a procedure currently used in this laboratory (14).

RESULTS :

1 . Formation of lactoperoxidase compound III at + 20° :

Since O_2^- reacts with lactoperoxidase ($Fe_p^{3+} - H_2O$) to give compound III ($Fe_p^{3+} - O_2^-$) (12) it was anticipated that if produced during the aerobic photosensitization of some dyes, O_2^- could be detected by the absorption spectrum of compound III. Figure 1 demonstrates that this was the case upon irradiation of fluorescein (10^{-5}M) at 480 nm in presence of the lactoperoxidase (10^{-5}M). Compound III is stable under the conditions used here and its characteristic absorption bands (546 and 588 nm) do not interfere with the measurement of the absorption spectrum of fluorescein ; but this is not true of other dyes which might be used as photosensitizers for O_2^- generation.

On the other hand aromatic molecules such as tryptophan and nucleotides (nicotinamide adenine diphospho nucleotide) absorbing respectively at 280 and 260 nm have been successfully used to form compound III, and the photo excitation of tryptophan residues of lactoperoxidase led to a similar result.

The yield of compound III by irradiation of fluorescein was 70 % of that obtained by the addition of 10^{-3}M H_2O_2. Since H_2O_2 in excess normally leads to the formation of compound III (15), we checked that the photoreaction was not due to the formation of H_2O_2 by the addition of catalase (5.10^{-7}M) when we observed the same yield of compound III, as shown in Figure 1.

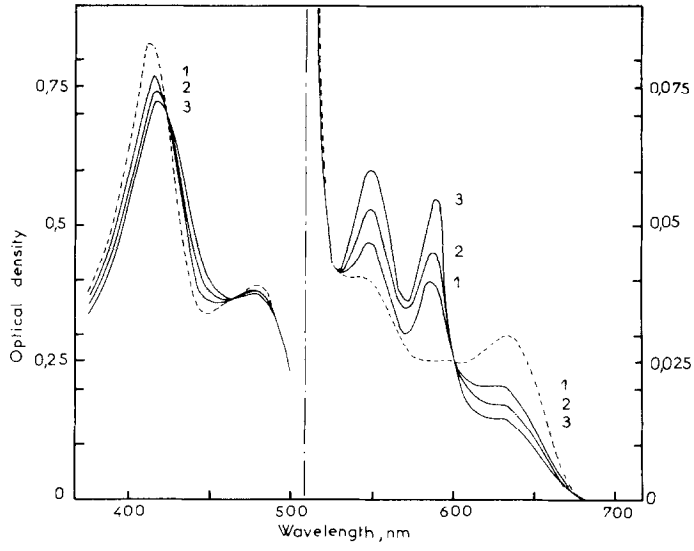

Figure 1 : Formation of compound III of 10^{-5}M lactoperoxidase upon irradiation of 10^{-5}M fluorescein, at room temperature in 0.1M cacodylate buffer pH 6.2.
. . . . . lactoperoxidase and dye before irradiation
——— after 1 min, 2 min and 5 min of irradiation for curves 1, 2 and 3 respectively.

2 . Formation of compound III at low temperatures :

The above reaction with fluorescein was also carried out in a fluid mixture of ethylene glycol and aqueous buffer (v/v = 1:1) at -40° and the formation of compound III was recorded with a 30 % lower yield and a rate of formation three times lower than under the above-mentioned normal conditions at room temperature. H_2O_2 could be excluded as a significant reactant by

our failure to observe compounds I and II, which would be stabilized at low temperature (16). The rate of formation of compound III was directly proportional to light intensity, both at low temperature and at room temperature, as shown in Figure 2.

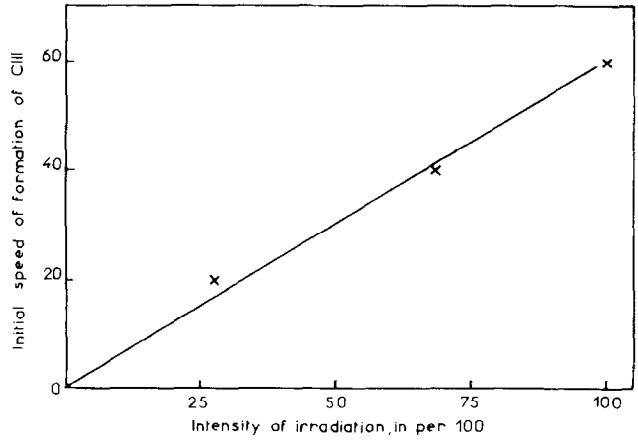

Figure 2 : Initial speed of formation of compound III (at 588 nm) as a function of irradiation intensity. Temperature + 20°.

3 . Inhibition of compound III formation by superoxide dismutase :

In aqueous buffered solutions (0.1M Tris buffer, pH 7.5), where the yield of compound III from 10^{-5}M lactoperoxidase was about 40 % after 5 minutes of irradiation, the presence of superoxide dismutase (5 µg/ml) lowered the yield to less than 8 %. Such an inhibition confirms the involvement of superoxide anions in the photoprocess leading to the formation of compound III.

DISCUSSION

The data presented above can be explained by the production of superoxide ions O_2^- during the photosensitized oxidation of fluorescein under aerobic conditions. The fact that the rate of formation of compound III (and thus the rate of production of O_2^-) is directly proportional to the light intensity, and not to its square, rules out a biphotonic electron ejection.

The following three processes have been previously taken into account to explain the photooxidative bleaching of dyes (D) :

$D^T + {}^3O_2 \longrightarrow D + {}^1O_2$ (possible interaction between triplet dye and oxygen) (process 1)

$D^T + {}^3O_2 \longrightarrow D^+ \ldots O_2^-$ (complex resembling an ion-pair) (process 2)

$D^T + {}^3O_2 \longrightarrow D^+ + O_2^-$ (formation of free O_2^-) (process 3)

The occurrence of process 1 has been fully established since the pioneer work of Foote and Wexler (17, 18). Process 2 has been suggested as a special case of the general adduct-theory of Schenk (19, 20). Process 3 was proposed by Weiss, Franck, Livingston and others (21). The three processes might occur simultaneously. The formulation of free O_2^- , and accordingly the occurrence of process 3 has been ruled out by Koizumi and Usui (11, 22) who took into consideration observations made with aqueous as well as ethanolic solutions. These authors postulated that the only possible process was the formation of the complex $D^+ \ldots O_2^-$ (process 2) most of which would revert to the initial dye and oxygen, while only a small portion (quantum yield 10^{-4}) would decompose irreversibly.

It can be seen in the present experiments, where lactoperoxidase was used as a scavengrer of O_2^- to form compound III, that the yield of O_2^- is not negligible since the yields of compound III can be compared to those classically obtained with an excess of hydrogen peroxide.

Finally, from a practical point of view, it appears that photosensitization might be used as a valuable O_2^- - generating process and lactoperoxidase as a detector of such ions. The fact that aromatic compounds such as nucleotides and amino acid residues can behave as O_2^- - generating systems when excited under aerobic conditions deserves much attention and a forthcoming paper will deal with this subject and its application to the study of photobiological problems.

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