Peroxidase-catalyzed co-oxidation of indole-3-acetic acid and xanthene dyes in the absence of hydrogen peroxide

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The effect of xanthene dyes on the chemiluminescence from the aerobic indole-3-acetic acid (IAA) oxidation, catalyzed by horseradish peroxidase (HRP), was studied. The rate of IAA oxidation and dye destruction were controlled. It was found that the addition of dyes to the IAA/HRP/O₂ system resulted in: (i) the appearance of emission in the region of dye fluorescence, (ii) an increase of the total chemiluminescence intensity, (iii) a decrease of the emission duration, (iv) the acceleration of IAA oxidation, and (v) slow bleaching of the dyes. The experimental results lead to the conclusion that all spectral and kinetic variations of the chemiluminescence from the IAA/HRP/O₂ system which are caused by the addition of xanthene dyes, are the result of IAA-dye co-oxidation. Earlier published reports regarding energy transfer from electronically excited species, generated in the IAA/HRP/O₂ system, to the xanthene dyes seem to be erroneous.

Horseradish peroxidase; Indole-3-acetic acid; Xanthene dyes; Co-oxidation

1. INTRODUCTION

Indole-3-acetic acid (IAA) is a natural phytohormone with many growth regulatory function. The level of IAA in plants is, in particular, controlled via its enzymatic oxidation that is catalyzed by peroxidases [1]. It is known that peroxidase-catalyzed aerobic oxidation of IAA gives rise to the formation of electronically excited products [2,3]. Cilento et al. widely studied the effect of the IAA/HRP/O₂ system on the different substances. It was found that t-RNA [4–6], DNA [5] and chlorophyll [7] being added to the above system underwent the conversions, which were similar to the photochemical ones. Moreover, the addition of some fluorescent dyes to the IAA/HRP/O₂ system resulted in the emission in the range of dye fluorescence [4,7,8]. These data were interpreted by the authors as the energy transfer from enzyme-generated electronically excited species to the added acceptors. This interpretation, however, has a number of drawbacks: particularly, the obtained values of the Stern–Volmer constants (10^4–10^5 M^{-1}) significantly exceed the possible one for the energy transfer to the fluorescent dyes in the O₂-containing solutions.

In the present work we carefully investigated the effect of xanthene dyes on the chemiluminescence from the IAA/HRP/O₂ system. The rate of IAA oxidation and dye bleaching were controlled. The observed spectral and kinetic variations of chemiluminescence are ascribed to the enzymatic co-oxidation of the IAA-dye pair.

2. MATERIALS AND METHODS

HRP (RZ 3.0), IAA, fluorescein, erythrosin B, rhodamine 6G, rose bengal, and the components of the phosphate buffer were obtained from Sigma. H₂O₂ was obtained from Aldrich. Solutions were prepared using triple distilled deionized H₂O. The standard reaction mixture contained 1 mM IAA, 0.1 μM HRP in 0.067 M phosphate buffer, pH 7.4. Unless otherwise stated, the fluorescent dye of a required concentration was added before the peroxidase addition. The final volume of the reaction mixture was 3 ml.

The spectrophotometric measurements were made with a double beam spectrophotometer 'Hitachi 557' (Japan) in quartz cuvettes with optical path length 1 cm. The formation of IAA oxidation products was observed by the measurement of the differential absorbance between 242 and 296 nm (points with equal values of IAA absorbance). The bleaching of the dyes in the HRP/H₂O₂ system was measured at the wavelength of maximal dye absorption. Under the study of the dye bleaching in the IAA/HRP/O₂ system the absorption of IAA oxidation products was taken into account.

The kinetics and spectra of chemiluminescence during the enzymatic reaction were followed with the specially designed chemiluminometer, slightly modified compared to that, which was previously reported [2]. First, six quartz cuvettes, each 30 mm in diameter, with reaction mixtures were arranged on a rotating disc driven by a computer-controlled stepping motor. Second, the correction of spectra for the spectral sensitivity of photomultiplier provided us with real quantum intensity values. In order to determine the intensity of the dye emission in the IAA/HRP/O₂/dye system we separated the total emission into four spectral components. Three components with the maxima at 420, 465 and 535 nm are the emission of the excited products of IAA oxidation [2], the fourth component is the dye fluorescence.

All experiments were carried out at 22°C.

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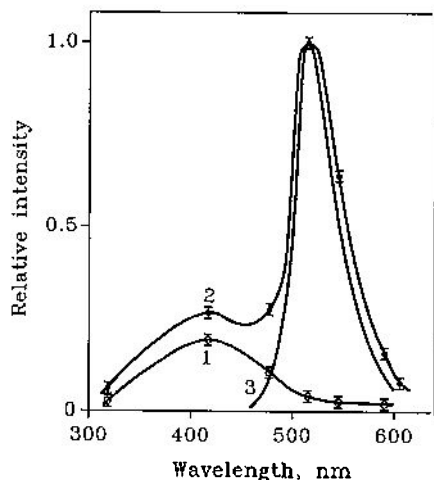
Fig. 1. The effect of fluorescein upon the chemiluminescence spectrum of the 1 mM IAA/0.1 μM HRP/O$_2$ system: (1) without fluorescein, (2) in the presence of 5 μM fluorescein, (3) fluorescein fluorescence spectrum for λ_{ex} = 442 nm (normalized on the maximum of chemiluminescence spectrum in the presence of fluorescein).
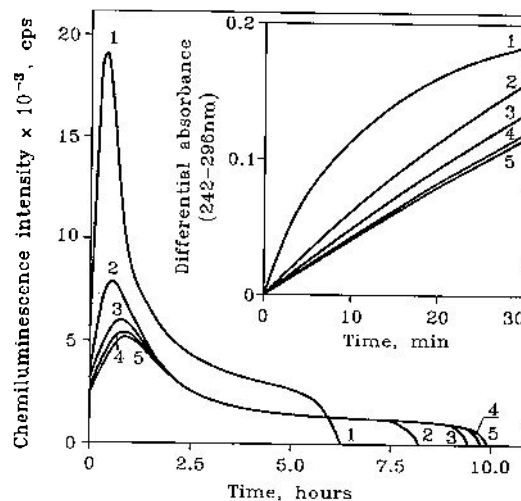

Fig. 2. The time dependence of the chemiluminescence intensity of the 1 mM IAA/0.1 μM HRP/O$_2$ system in the presence of 5 μM xanthene dyes. The inset figure shows the effect of 5 μM xanthene dyes on the kinetics of the IAA oxidation in the above system. Dyes: (1) fluorescein, (2) erythrosin B, (3) rose bengal, (4) rhodamine 6G, (5) without any dye.

3. RESULTS AND DISCUSSION

Firstly the effect of xanthene dyes on the chemiluminescence from the IAA/HRP/O$_2$ system was studied. We observed an appearance of emission in the dye fluorescence region (see for example Fig. 1) along with an increase of integral quantum intensity of the emission and decrease of the total time of the light reaction (Fig. 2). Spectrophotometric measurements showed that xanthene dyes caused an acceleration of the IAA oxidation (Fig. 2, inset). Moreover, slow bleaching of the dyes was observed during the IAA oxidation (see for example Fig. 3). No detectable dye bleaching was found in the presence of HRP without IAA. The presence of IAA without HRP also did not result in the dye bleaching. So the IAA-dye pair was likely to undergo a co-oxidation in the IAA/HRP/O$_2$ system.

The presence of IAA is essential for the dye oxidation because IAA causes the transition of HRP from the inactive ferric form (Fe$_p^{3+}$) to the active forms (Co-I, Co-II, Co-III or Fe$_p^{3+}$) [9]. It is well known that efficient Co-I formation can be brought out by the addition of H$_2$O$_2$ to the initial Fe$_p^{3+}$ form [10]. This is why we studied further the influence of HRP/H$_2$O$_2$ system on the xanthene dyes. It was found that the dyes were relatively quickly bleached in the presence of HRP and H$_2$O$_2$ (Fig. 4, inset). Moreover the dye oxidation was accompanied by the intensive chemiluminescence (Fig. 4). The spectra of this emission were identical to those of the dye fluorescence.

Using fluorescein (the most active dye) we have compared the quantity of emitted quanta (at the wavelength of the dye fluorescence) per mol of bleached dye for the IAA/HRP/O$_2$/dye and HRP/H$_2$O$_2$/dye systems after the

cessation of chemiluminescence in the IAA/HRP/O$_2$/dye system. These values were: $\varphi_1 = (4 \pm 0.4) \cdot 10^{14}$ in the first system and $\varphi_2 = (4.2 \pm 0.1) \cdot 10^{14}$ in the second system. They are equal within the limits of experimental errors. It should be noted that all chemiluminescence from the HRP/H$_2$O$_2$/dye system is obviously the result of the dye oxidation. Taking these facts into account we can conclude that the emission from the IAA/HRP/O$_2$/dye system in the dye fluorescence region is also caused by the dye oxidation only. The energy transfer from the electronically excited products of IAA oxidation to the xanthene dyes is probably absent because the energy transfer would result in the apparent increase of the φ_1 value in comparison to φ_2.
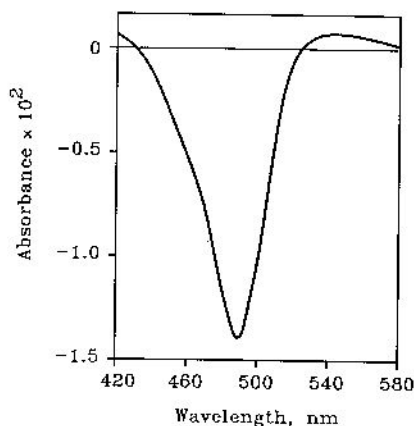

Fig. 3. Difference between absorption spectra of fluorescein after and before the IAA oxidation in the 1 mM IAA/0.1 μM HRP/5 μM fluorescein/O$_2$ system.
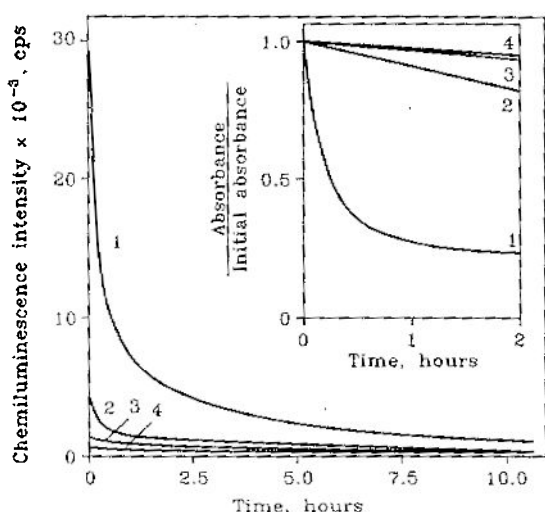
Fig. 4. Chemiluminescence during the oxidation of 5 μM xanthene dyes in the 0.1 μM HRP/0.1 μM H_2O_2 system. The inset figure shows the bleaching of 5 μM xanthene dyes in the above system (the absorbance was measured at the maximum of dye absorption). Dyes: (1) fluorescein, (2) erythrosin B, (3) rose bengal, (4) rhodamine 6G.

So the increase of total chemiluminescence intensity with the addition of dyes to the IAA/HRP/O_2 system is the result of the IAA oxidation acceleration and the chemiluminescence during the dye oxidation. The emission in the dye fluorescence range appears only because of the dye oxidation. The decrease of the light reaction duration is caused by the IAA oxidation acceleration. Kobayashi et al. [11] ascribed the shortened emission in the presence of dyes to the dye destruction. However, this suggestion seems to be unlikely. First, the IAA/HRP/O_2 system emits without dye (Fig. 2, curve 5), that is, the cessation of chemiluminescence is the result of the end of emitting reaction stages. Second, the proportion of bleached dye after the end of IAA oxidation does not exceed 4% even for the most active dye, fluorescein.

To summarize, our results lead to the conclusion that all spectral and kinetic variations of the chemiluminescence, caused by the addition of xanthene dye to the IAA/HRP/O_2 system, are the result of enzymatic cooxidation of the IAA-dye pair. Some previous conclusions [4,8] concerning the energy transfer from enzyme-generated electronically excited species to these dyes are most likely unfounded. Therefore, the numerous reports (see for review [12–15]) on the energy transfer in the above system should be reexamined.

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