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GENERATION OF BIO-ELECTRONIC ENERGY BY ELECTRON TRANSFER: REDUCTION OF PEROXIDASE COMPOUND I AND COMPOUND II BY EOSINE

Peter J.O'Brien[+], Etelvino J.H. Bechara, Cindy R.O'Brien[++], Nelson Duran[+++] and Giuseppe Cilento*

Instituto de Química, Department of Biochemistry, Universidade de São Paulo, C.P. 20780, São Paulo, Brazil.

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SUMMARY: The spontaneous reduction of peroxidase Compound I is accompanied by a very weak emission which is greatly magnified by eosine. A further, dramatic enhancement is observed in the presence of certain tertiary aliphatic diamines. The chemiluminescence spectrum corresponds to eosine fluorescence. This is the first case of significant formation of electronically excited states by electron transfer in a biochemical system.

The generation of electronically excited states in high yields in biochemical systems other than those which are bioluminescent is under active investigation in our laboratories (1-16). Most of the systems in which we have previously detected the generation of an excited product are reactions which can, at least formally, proceed through a dioxetane intermediate. In all cases, the enzyme is an ironporphyrin protein, usually a peroxidase.

This paper reports the formation of excited states in a system of peroxidase, H_2O_2 and eosine, both in the absence and in the presence of amines.

+ Visiting Professor from Memorial University of Newfoundland under the National Research Council of Canada/Conselho Nacional de Pesquisas do Brasil Program.
++ Science Assistant from Memorial University of Newfoundland.
+++ FAPESP Visiting Professor from Universidad Catolica de Valparaiso, Chile.
* To whom inquires should be addressed.

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MATERIALS AND METHODS

Horseradish peroxidase (HRP; type VI) was obtained from Sigma, eosine was from Merck and 9,10-dibromo anthracene-2-sulfonate (sodium salt) was synthesized (17). All other chemicals were the purest grade commercially available. Hydrogen peroxide, 30% by weight, was obtained from Merck and diluted before use. Its concentration was determined by the peroxidase catalyzed oxidation of iodide (18). All reactions were carried out at 25°C in acetate or phosphate buffer and started by addition of H_2O_2.

Absorption spectra were taken on a Zeiss DMR-21 recording spectrophotometer using 1 cm cells. The chemiluminescence was measured in a Beckman LS-250 Liquid Scintillation Counter, in a Hamamatsu TV Photoncounter C-767 (19) and, when strong enough in a Hitachi-Perkin Elmer MPF-4 Spectrophotometer. The quantum yield of emission was measured using as a standard the scintillation "cocktail" as described previously (11). The chemiluminescence emissions were initially recorded in counts per 5 seconds and converted to emission intensities by the experimentally established conversion factor 2.7×10^{-21} einstein/counts,ml. minute. The fluorescence quantum yield of eosine in water was also measured (20).

RESULTS AND DISCUSSION

The addition of H_2O_2 to HRP results in a very weak chemiluminescence (21). The emission is enhanced in the presence of eosine or fluorescein but not by anthracene-2-sulfonate, 9,10-dibromoanthracene-2-sulfonate or riboflavin. The total emission chemiluminescence in the presence of eosine was directly proportional to the concentration of peroxidase (1-5 μM) and was optimal at pH 5.8 in acetate buffer. No chemiluminescence was observed in the absence of H_2O_2 or peroxidase. Cyanide ion, being a peroxidase inhibitor, suppressed the emission.

It is known that phenols can reduce HRP-I to HRP-II (22). In our system eosine is partially bleached and therefore is clearly the reductant. Fig. 1 shows that bleaching correlates with the disappearance of HRP-I. Fig. 1 also shows that emission follows the disappearance of HRP-II; therefore, the species gen-

ABBREVIATIONS: HRP, horseradish peroxidase; TMED, N,N,N',N'-tetramethylethylenediamine.

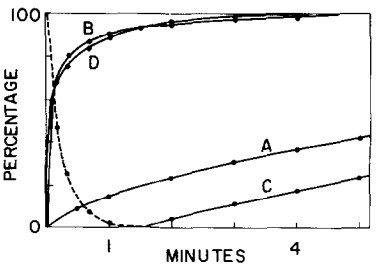

Fig. 1 - Correlation of the integrated photon emission (A) with the decay of HRP-I (B), decay of HRP-II (C) and eosine bleaching (D). The decay of HRP-I was followed by the increase of absorbance at 411 nm. The decay of HRP-II was monitored by the decrease of absorbance at 420 nm; the dotted part of curve C therefore represents the formation of HRP-II. Eosine bleaching (curve D) was recorded as loss of absorbance at 517 nm. The following concentrations were used: HRP, 4.2 μM; H_2O_2, 4.2 μM; eosine, 20 μM. The buffer was 0.10 M phosphate, pH 6.2.

erated excited is possibly HRP, which would be formed by electron addition to an upper vacant orbital of Fe (IV) in the iron-porphyrin of HRP-II.

Striking results, which proved to be more informative, were obtained in the presence of the tertiary aliphatic diamines EDTA and TMED. These two amines, in contrast to the other amines assayed, greatly increase the rate of photon emission by the system HRP-H_2O_2 - eosine (Fig. 2). The observation of a maximum in Fig. 2, even in the absence of amine, indicates that the emission comes from an intermediate, i.e. from the HRP-II disappearance referred to above. Since the amine does not appreciably shift the maximum, it may be inferred that the amine must affect both the formation and the disappearance of HRP-II equally. Parallel experiments have, in fact, shown that the amine efficiently accelerates both the decay of HRP-I and the disappearance of HRP-II to a similar extent; again, as expected, the integrated emission roughly followed the disappearance of HRP-II. It should be noted that certain nitrogenous ligands are known to accelerate

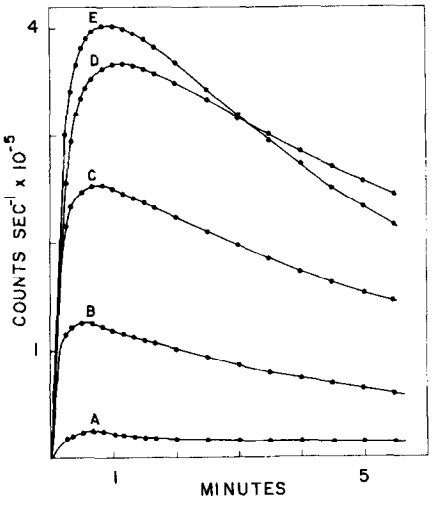

Fig. 2 - The effect of TMED and EDTA upon the rate of photon emission of the system 2.5 μM HRP - 1.5 mM H_2O_2 - 45 μM eosine in 0.10 M phosphate buffer, pH 6.2. A, no amine; B, 0.12 mM TMED; C, 0.50 mM TMED; D, 1.0 mM TMED; E, 0.50 mM EDTA. No effect was observed with the following amines (0.50 mM): 1,4-diazabicyclo[2.2.2]octane, triethylamine, piperidine, diethylamine, n-pentylamine, sec-butylamine.

reactions of peroxidase (23). However, these two amines act specifically in our system.

The increase in emission in the presence of the amine is related to the increased rate of reaction. Furthermore, eosine bleaching is diminished considerably (completely absent with 10-20 mM TMED); therefore, eosine is available to act as both reductant and sensitizer. Optimizing the conditions, one can obtain the chemiluminescence spectrum even with conventional equipment, the spectrum being identical to eosine fluorescence (Fig. 3).

Comparison with the chemiluminescence spectrum in the absence of amine thus became relatively important. Using a large excess of eosine and the technique employed by Nakano et al (24), the spectrum from the reaction in phosphate buffer was determined and found to be quite similar to that reported in Figure 3.

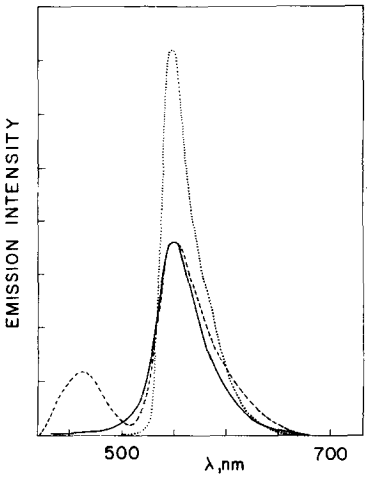

Fig. 3 - Chemiluminescence spectrum of the system 2.5 μM HRP - 1.5 mM H_2O_2 - 90 μM eosine - 1.0 mM TMED at 25°C: _____ in 0.10 M acetate, pH 5.6; ---------- in 0.10 M phosphate, pH 6.2. The curve .....,is the fluorescence spectrum (λ_{exc} = 480 nm) of the system (in acetate) before addition of $H_2O_2$and coincides with that of eosine. A large excess of $H_2O_2$was used to insure determination of the chemiluminescence spectrum under steady state conditions. The peak at 460 nm must be ascribed to a transient species because it does not appear in the fluorescence spectrum of the spent reaction mixture.

Therefore, the amine has no influence on the quality of the light emitted.

The following mechanism

$$HRP\text{-}I + R\text{-}O^- \longrightarrow HRP\text{-}II + R\text{-}O^{\cdot} \qquad \text{step 1}$$

$$HRP\text{-}II + R\text{-}O^- \longrightarrow HRP^* + R\text{-}O^{\cdot} \qquad 2$$

$$HRP^* + R\text{-}O^- \longrightarrow HRP + {}^1R\text{-}O^{-*} \qquad 3$$

$${}^1R\text{-}O^{-*} \longrightarrow R\text{-}O^- + h\nu \qquad 4$$

$$R\text{-}O^{\cdot} \longrightarrow \longrightarrow \longrightarrow \text{bleached products} \qquad 5$$

$$R\text{-}O^{\cdot} + R_3N: \longrightarrow R\text{-}O^- + R_3N.^+ \qquad 6$$

where $R\text{-}O^-$ represents eosine, is tentatively proposed to account for our results. EDTA and TMED should not be acting as reductants of HRP-II because they exhibited no effect whatsoever on HRP-I

when eosine was absent. This is in contrast to the behavior of tertiary aromatic amines (25).

The alternative that the eosine radical is the species which is generated excited in step 2 should be considered. The possibility, although very interesting in its own right, is less likely since no excitation is generated in step 1. Another alternative, generation of excited eosine in step 6, can be dismissed on energetic grounds.

Based on the hydrogen peroxide added to the system, the observed quantum yield of emission - whether the amine is present or not - is about 7×10^{-8} einstein/mole. The yield of chemi-excitation may be orders of magnitudes higher because (i) the quantum yield of eosine fluorescence is 0.2 (ii) H_2O_2 may have disappeared by other routes (iii) the efficiency of energy transfer to eosine may be low in view of other competing processes; we note for example, that, in high spin ironporphyrin proteins, conversions between states of different multiplicities are greatly favoured.

Independent of the precise mechanistic details of the process, the present work conclusively demonstrates that there may be efficient generation of excited electronic states as a result of electron transfer in a biochemical system. The very interesting possibility arises that, when peroxidase acts peroxidatically, it is the enzyme itself which may become electronically excited; on the other hand, when the peroxidase acts as an oxidase, the product may be generated electronically excited, provided that the substrate is appropriately chosen (15).

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