SPECTRAL ANALYSIS OF THE LOW LEVEL CHEMILUMINESCENCE OF H_2O_2-SUPPLEMENTED FERRICYTOCHROME *c*

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1. Introduction

The role of ferricytochrome *c* as an initiator of oxidative free radical reactions leading to lipoperoxidation has been widely described [1–3]. The participation of hemoproteins in lipid autoxidation and lipid peroxide breakdown has been reported to be accompanied by chemiluminescence; the spectral distribution of the light emission observed in the autoxidation of sodium linoleate [4–5] was not modified when the reaction was accelerated by either cytochrome *c* or hemoglobin [6]. Hawco et al. [7] observed photoemission from the hematin-catalysed decomposition of linoleic acid hydroperoxide and suggested the participation of singlet oxygen in the process.

We have described the chemiluminescence of cytochrome *c*/organic hydroperoxide mixtures as well as the importance of cytochrome *c* in the chemiluminescence of mitochondria and submitochondrial particles [8,9] and presented evidence of the participation of singlet molecular oxygen in these systems.

Here we report on the photoemission of H_2O_2-supplemented ferricytochrome *c* and on the nature of light emission as studied by spectral analysis and the effect of chemiluminescent singlet oxygen quenchers.

2. Materials and methods

2.1. *Chemicals*

Cytochrome *c* (type VI) and myoglobin were obtained from Sigma Chemical Co. (St Louis, MO); cytochrome *c* was used without further purification. β-Carotene, 1,4-diazabicyclo-[2,2,2]-octane (DABCO) and *t*-butyl hydroperoxide (*t*-BuOOH) were purchased from Aldrich Chemical Co. (Milwaukee, WI); H_2O_2 was from Baker Chemical Co. (Phillipsburg, NJ). Other reagents used were of analytical grade.

2.2. *Photon counting*

A red-sensitive EMI photomultiplier, responsive in the range 300–900 nm, was connected to a power supply (– 1.2 kV) and fed into a Princeton Applied Research (Princeton, NJ) amplifier–discriminator (model 1121) adjusted for single photon counting [10,11]. Spectral distribution of chemiluminescence was carried out by sliding a wedge interference filter (Schott Optical Glass Co., FRG) into a channel running between the cuvette holder and the photomultiplier. The interference filter was moved manually every 0.25 cm and the total chemiluminescence yield calculated for every reaction at that wavelength; a similar device was described in [12]. The light transmitted by the interference filter was expressed as percentage of the total light detected by the photomultiplier after correcting for the quantum efficiency corresponding to each wavelength by the following formula:

$$QE = \frac{\sigma\,(1239.5)\,(100)}{\lambda}\ \%$$

where σ is the cathode radiant sensitivity in A/W at a wavelength λ in nm.

2.3. *Assay conditions*

All the assays were carried out in 0.1 M potassium phosphate buffer (pH 7.2–7.3) at 37°C in a 8.0 cm^2 surface cuvette with 4 ml final vol.

3. Results

3.1. *Chemiluminescence of H_2O_2-supplemented ferricytochrome* c *mixtures*

Addition of H_2O_2 to a buffered solution containing ferricytochrome *c* showed low level chemiluminescence (fig.1). The chemiluminescent signal increased immediately after the addition of H_2O_2 to ferricytochrome *c*, achieving the maximal light emission yield in ~1–2 min, decaying more slowly to the background level. The insets in fig.1 show the dependency of the H_2O_2/ferricytochrome *c* light emission on cytochrome *c* [inset (a)] and H_2O_2 [inset (b)] concentrations. Photoemission was a linear function of the cytochrome *c* concentration at high H_2O_2 concentration and showed a tendency to saturation when dependent on H_2O_2 concentration. At low H_2O_2 concentration no light emission was observed, presumably due to the antioxygenic effect of cytochrome *c* [13].

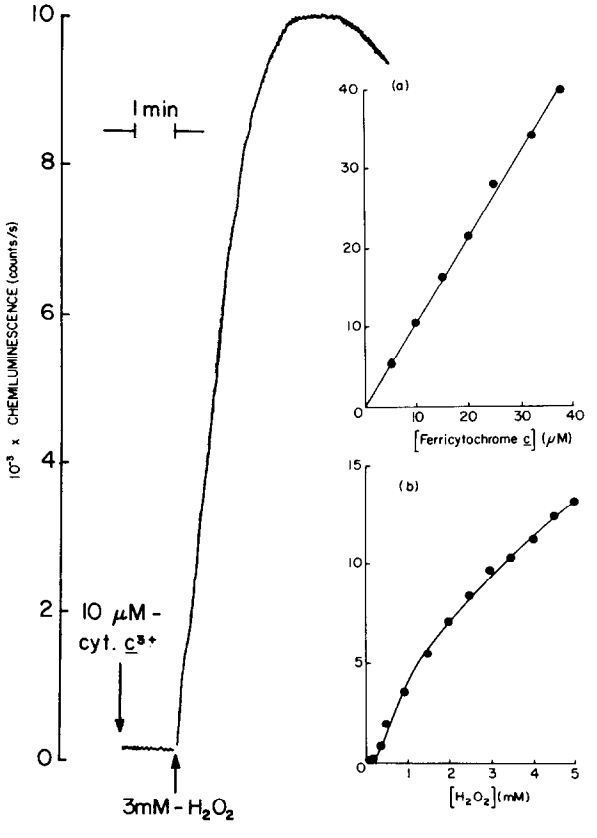

Fig.1. Chemiluminescence of H_2O_2-supplemented ferricytochrome *c*. Assay conditions described in section 2. Chemiluminescence signal of ferricytochrome *c* (cyt. c^{3+}) upon addition of H_2O_2. Insets: (a) chemiluminescence against ferricytochrome *c* concentration in the presence of a constant amount of H_2O_2 (3 mM); (b) chemiluminescence against H_2O_2 concentration in the presence of a constant amount of ferricytochrome *c* (10 μM).

3.2. *Spectral distribution of the chemiluminescence of cytochrome* c/H_2O_2 *mixtures*

Identification of the source of photoemission in the present system was approached by chemical (effects of a quencher and an enhancer of singlet oxygen chemiluminescence) and physical (spectral distribution of light emission) analysis of the reaction.

The effect of β-carotene as an effective quencher of singlet oxygen was described in [14]. Fig.2 shows the inhibitory effect of β-carotene on the light emission of H_2O_2-supplemented cytochrome *c* with a half maximal effect at 0.3 mM β-carotene. Dimethylsulfoxide or methanol, the solvents used for β-carotene, affect neither the kinetic nor the total yield of the chemiluminescence reaction.

On the other hand, 1,4-diazabicyclo-[2,2,2]-octane is an enhancer of singlet oxygen dimol emission in

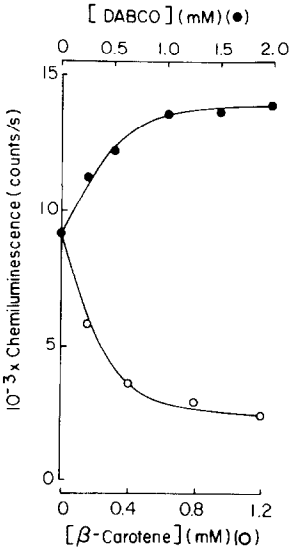

Fig.2. Effect of β-carotene and 1,4-diazabicyclo-[2,2,2]-octane on light emission of H_2O_2-supplemented cytochrome *c*. Assay conditions as described in section 2. Incubation mixture contained 10 μM ferricytochrome *c* supplemented with 5 mM H_2O_2 in the absence (control) or presence of various concentrations of β-carotene (○) or 1,4-diazabicyclo-[2,2,2]-octane (DABCO) (●).

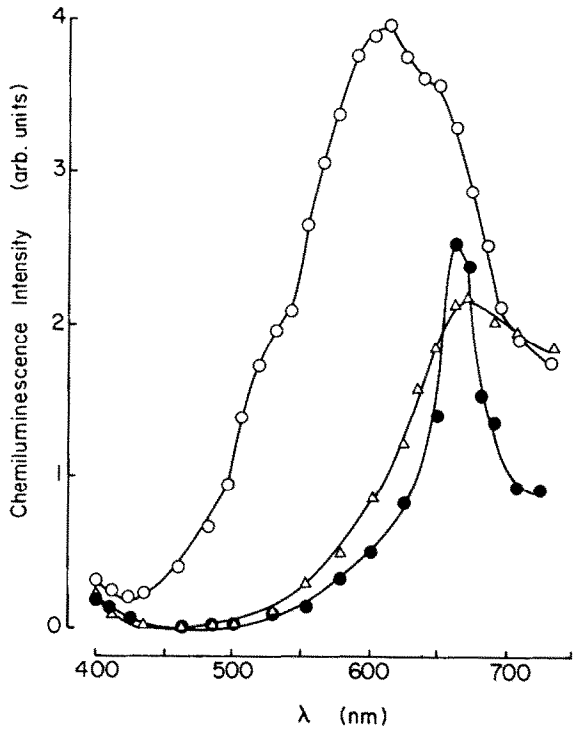

Fig.3. Spectral analysis of the chemiluminescence of hydroperoxide/hemoprotein systems. Spectral distribution of chemiluminescence was measured with a wedge interference filter as explained in section 2. Chemiluminescence spectrum of: (○) ferricytochrome *c* (50 μM) + H_2O_2 (10 mM); (●) myoglobin (125 μM) + *t*-butyl hydroperoxide (10 mM); (△) ferricytochrome *c* (125 μM) + *t*-butyl hydroperoxide (10 mM).

aqueous solutions [15,16]; in our system it increased light emission by a factor of ~1.5 at 1.5–2.0 mM. It is worth noting that the model system containing *t*-butyl hydroperoxide/cytochrome *c* [9] as well as the hydroperoxide-supplemented submitochondrial particles [8] needed a 5–10-times higher concentration of DABCO to enhance chemiluminescence by a factor of 2. This high concentration of DABCO was similar to that in [16] in the model system containing hypochlorite and H_2O_2.

The spectral analysis of the chemiluminescence of H_2O_2-supplemented cytochrome *c* showed a distribution entirely in the visible region with a main peak at ~600–612 nm and two shoulders around 519–531 nm and 638–650 nm (fig.3). The spectrum observed seemed dependent more on the type of hydroperoxide utilized than on the nature of the hemoprotein. The breakdown of the tertiary hydroperoxide, *t*-butyl hydroperoxide, catalyzed by either cytochrome *c* or myoglobin, produced chemiluminescence spectra with main peaks at 660–680 nm (fig.3). The total chemiluminescence yield of H_2O_2-supplemented cytochrome *c* was 10-times bigger than the *t*-butyl hydroperoxide supplemented cytochrome *c*; this allowed a better resolution of the spectrum, as can be seen in fig.3.

4. Discussion

The chemiluminescence of hydroperoxide-supplemented hemoproteins affords a model system applicable to biological membranes [8,9]; cytochrome *c* and other hemoproteins are widely distributed and, on the other hand, the cellular generation of H_2O_2 has been proved to take place in most aerobic cells (reviewed [17]).

Chemiluminescence arising from this system primarily might be due to singlet oxygen dimol emission or to a dioxetane mechanism [18]; this will provide essentially red band chemiluminescence (singlet oxygen dimol emission) [19] and green band chemiluminescence (dioxetane mechanism) [18].

The participation of singlet oxygen in our system primarily could be suggested by the effects of β-carotene and DABCO. Although several criticisms regarding the specificity of the singlet oxygen quenchers, traps and enhancers have been reasonably pointed out [20], the effects of β-carotene and DABCO still have strong documented evidence [14–16].

The red chemiluminescence bands assigned to the transition of the oxygen molecule from the first excited state to the ground state ($2[^1\Delta g] \rightarrow 2[^3\Sigma\bar{g}]$ transition) for the reaction of H_2O_2 with hypochlorite, primarily the 634 and 703 nm bands [19,21–22] seem to be slightly shifted toward shorter wavelength (600–612 nm) in the H_2O_2-supplemented cytochrome *c* system, and toward longer wavelength (662–670 nm) in the *t*-butyl hydroperoxide-supplemented cytochrome *c* or myoglobin. The failure to detect a maximal light emission at 634 or 703 nm could be due to a low resolution of our system or to the heterogeneity of the chemiluminescence reaction; in this connection, a shift of singlet oxygen emission in the system hypochlorite/H_2O_2 obtained by addition of amino acids, albumin, or guanosine has been reported in [24].

Acknowledgements

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