LOW LEVEL CHEMILUMINESCENCE OF THE CYTOCHROME *c*-CATALYZED DECOMPOSITION OF HYDROGEN PEROXIDE

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1. Introduction

Hydroperoxides supplemented with hemoproteins are effective sources of photoemission. Hydrogen peroxide [1] and organic hydroperoxides (*t*-butyl, ethyl, and cumene hydroperoxide) [2] are comparatively active, though H_2O_2 shows a 10-fold higher chemiluminescence yield [1,2]. We have identified singlet molecular oxygen as the main chemiluminescent species in mixtures of cytochrome *c* with either H_2O_2 [1] or *t*-butyl hydroperoxide [2]. Considering that H_2O_2 is widely produced in cells and tissues [3] and that its interaction with hemoproteins may well explain part of the chemiluminescence observed in isolated cells and intact organs [4–7], we report here some of the properties of the chemiluminescence of the H_2O_2/cytochrome *c* system along with the effect of cyanide, radical traps and heavy metals.

2. Materials and methods

2.1. *Chemicals*

Cytochrome *c* (type VI) and superoxide dismutase were from Sigma Chemical Co. (St. Louis, MO); 2,5-di-*t*-butyl quinol was from Aldrich Chemical Co. (Milwaukee, WI); H_2O_2 from J. T. Baker Chemical Co. (Phillipsburg, NJ). Other reagents were of analytical grade.

2.2. *Photon counting*

Chemiluminescence was measured as in [1,2,4–7] with a 9658A red-sensitive EMI photomultiplier, responsive over 300–900 nm. Light emission was expressed in counts/s; 1 count/s corresponds to ~10 photons/s.

2.3. *Spectrophotometric assays*

Cytochrome *c* was used without further purification and its concentration was calculated by the A_{550} of ferrocytochrome *c* ($\epsilon_{550} = 27.7\ M^{-1} \cdot cm^{-1}$) [8]. The effect of H_2O_2 on cytochrome *c* heme was followed by the absorbance in the Soret band ($\epsilon_{408} = 109.5\ M^{-1} \cdot cm^{-1}$) [9] and initial rates expressed as nM cytochrome *c*/s.

Content of Cu^{2+} and Mn^{2+} in cytochrome *c* solutions was estimated by flame spectroscopy with a Varian (AA-575 series) atomic absorption spectrophotometer. Cu–amino acid complexes were prepared as in [10].

3. Results

3.1. *Chemiluminescence and spectroscopic changes of H_2O_2-supplemented ferricytochrome* c

The chemiluminescence observed upon addition of H_2O_2 to an aerobic solution of ferricytochrome *c* [1] (fig.1a) was accompanied by a decrease in the absorption bands of ferricytochrome *c* (fig.1b) due to heme destruction [11,12]. The 408 nm Soret band decreased rapidly and immediately after the supplementation of H_2O_2 to cytochrome *c*, at a rate of ~40 nM cytochrome *c*/s; the chemiluminescence peak was reached at the same time when almost a total decrease of the 408 nm band was recorded. H_2O_2 is ~10-times more effective in generating chemiluminescence than *t*-butyl hydroperoxide [2], and ~2-times more effective in generating the radicals that lead to heme destruction as estimated by the initial rates of the Soret band bleaching [2]. Apparently, reactions characteristic of the H_2O_2/cytochrome *c* system are more effective in generating the chemiluminescent species.

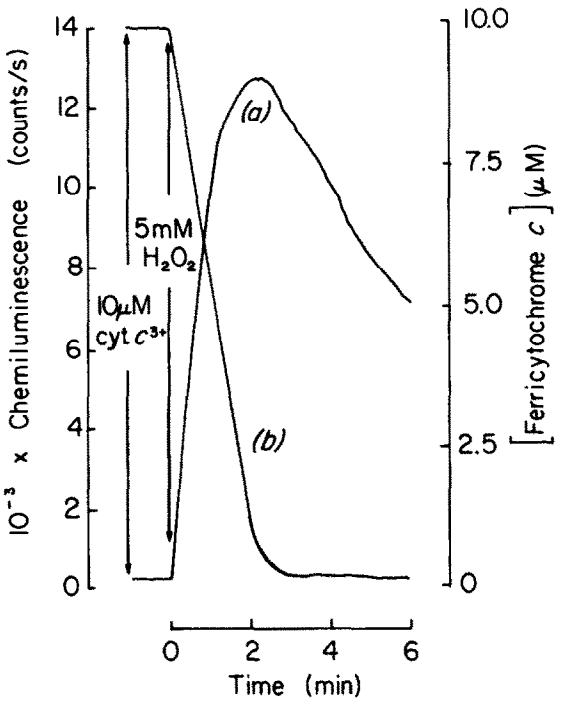

Fig.1. Chemiluminescence and spectroscopic changes of H_2O_2-supplemented cytochrome *c*. Chemiluminescence (a) and spectroscopic changes (followed at 408 nm) (b) as described in section 2. Assays were performed in 0.1 M potassium phosphate buffer (pH 7.2–7.3) at 37°C.

3.2. *Effect of cyanide on chemiluminescence of H_2O_2/cytochrome c mixtures*

Light emission and the decrease in the 408 nm band were inhibited effectively in ~87% by cyanide; half-maximal inhibitory effect was found at 0.9 mM cyanide (fig.2a); ~7-times higher concentrations of cyanide were necessary to obtain similar effects when H_2O_2 was present as compared with *t*-butyl hydroperoxide/cytochrome *c* mixtures [2]. Cyanide competed with H_2O_2 for cytochrome *c* as shown in fig.2b,c.

3.3. *Effect of Cu^{2+} and Mn^{2+} on chemiluminescence*

Taking into account that small amounts of Cu^{2+} and Mn^{2+} as contaminants of cytochrome *c* samples might lead to light emission upon reaction with H_2O_2, and that the inhibitory effect of cyanide might be due to the binding of the inhibitor to these metals, we assayed the effect of Cu^{2+} (free or as Cu–amino acid chelates) and Mn^{2+} on the chemiluminescence reaction. In our samples the molar ratio of contaminant Mn^{2+} or Cu^{2+} to cytochrome *c* was of ~0.0014. Both cations inhibited the photoemission of H_2O_2/cytochrome *c* mixtures by ~50% (fig.3). Cu–amino acid chelates (with histidine, tyrosine, or lysine) had the same effect for the case of the Cu–histidine chelate, where twice the concentration for a half-maximal effect was needed. Mn^{2+} and Cu^{2+} also inhib-

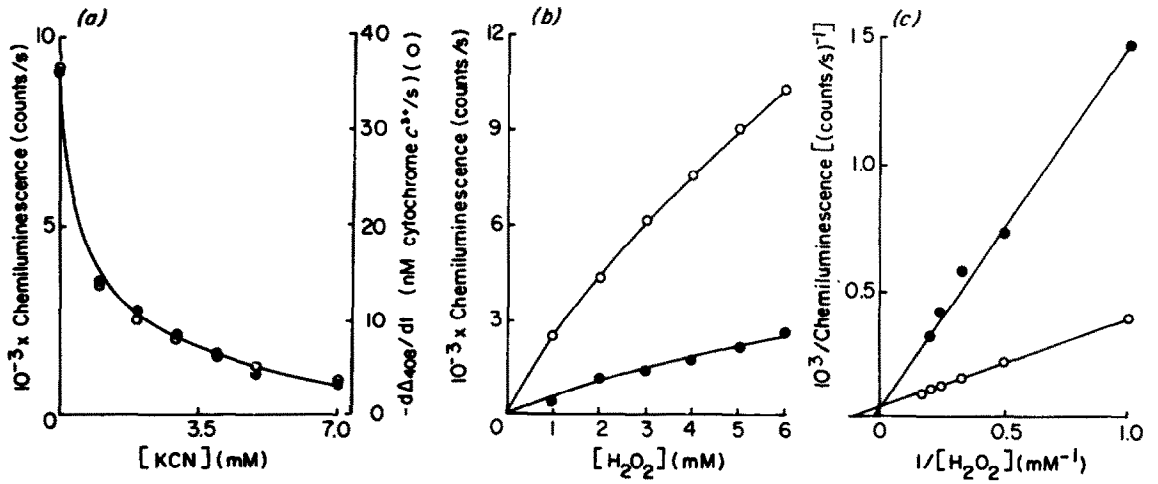

Fig.2. Effect of cyanide on chemiluminescence of cytochrome c/H_2O_2 mixtures: (a) Chemiluminescence (•) and rate of decrease of the A_{408} band (○) of a mixture containing 10 μM cytochrome *c* and 5 mM H_2O_2; (b) chemiluminescence of a 10 μM solution of cytochrome *c* supplemented with different amounts of H_2O_2 in the absence (○) and presence (•) of 7 mM cyanide; (c) reciprocal plot with data of (b). Experimental conditions as in fig.1.

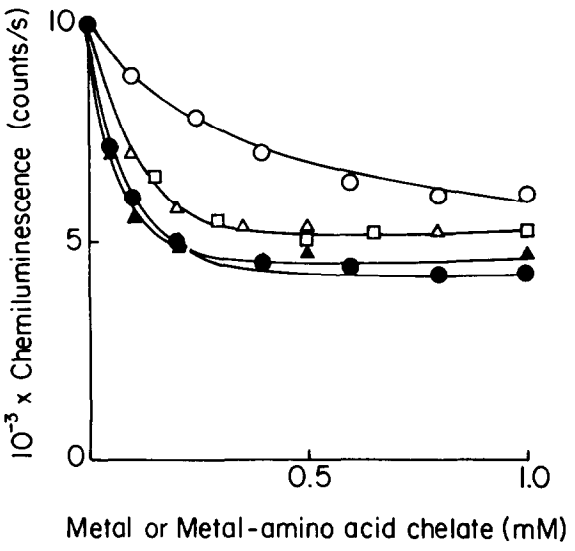

Fig.3. Effect of Cu^{2+} and Mn^{2+} on chemiluminescence of cytochrome c/H_2O_2 mixtures. The assay mixture contained 10 μM cytochrome c and 5 mM H_2O_2 (control). Effect of (•) Cu^{2+}, (○) Cu–lysine, (△) Cu–histidine, (□) Cu–tyrosine, (▲) Mn^{2+}. Experimental conditions as in fig.1.

ited t-butyl hydroperoxide/cytochrome c chemiluminescence [2]. Both cations, at 0.5–1.0 mM, did not affect the rate of cytochrome c Soret band bleaching.

3.4. *Free radicals in the H_2O_2-supplemented cytochrome c system*

Di-t-butylquinol is a very effective radical trap [13] as it proved to be in the tertiary hydroperoxide/cytochrome c system [2]. Di-t-butylquinol inhibited the total chemiluminescence yield, the rate of chemiluminescence, and the decrease in A_{408} (fig.4a,b). The 3 parameters were found ~60–70% decreased in the presence of 1 mM di-t-butylquinol.

Alcohols are known scavengers of HO· [14]; a series of them (methanol, ethanol, ethanediol, glycerol, propilenglycol, butanol, mannitol) was assayed at 25 and 250 mM, and they were found without effect on cytochrome c/H_2O_2 chemiluminescence. Superoxide dismutase (10 and 100 μg/ml) was also ineffective on cytochrome c/H_2O_2 chemiluminescence.

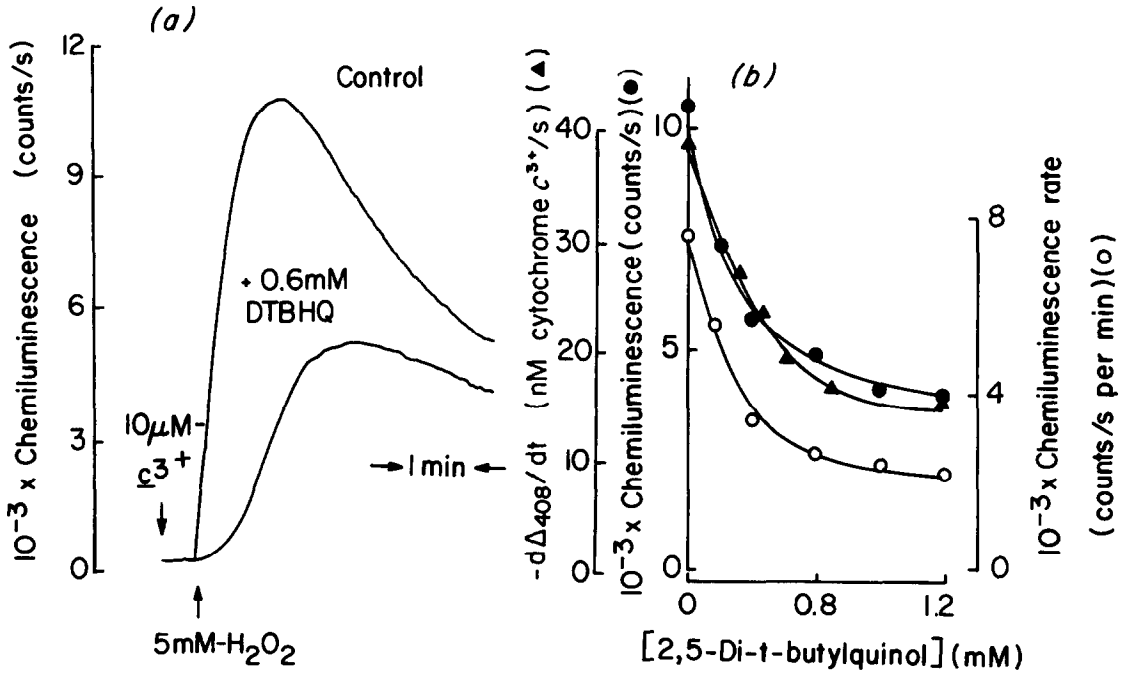

Fig.4. Effect of di-t-butylquinol on chemiluminescence and spectroscopic changes of cytochrome c/H_2O_2 mixtures. (a) Chemiluminescence signal in the presence and absence of di-t-butylquinol (DTBHQ); (b) effect of di-t-butylquinol on total chemiluminescence yield (•), rate of chemiluminescence (○), and decrease of A_{408} (▲).

4. Discussion

This paper deals with the mechanism of generation of the chemiluminescent species in the H_2O_2/cytochrome *c* system which was identified as singlet molecular oxygen in [1]. Singlet oxygen generation seems to include both, (a) reactions involving solely oxygen intermediates with a catalytic role for cytochrome *c* heme iron and, (b) radical reactions that lead to peroxidation and destruction of the cytochrome *c* molecule.

Reactions (1–3), which are supposed to occur in the vicinity of the heme group, are probably the best explanation for the observed effects, on the ground of the evidence of $HO^{\cdot}$ formation by the decomposition of H_2O_2 by Fe^{3+} complexes [15]:

$$c\text{–}Fe^{3+} + H_2O_2 \rightarrow c\text{–}Fe^{3+}\text{–}HO^{\cdot} + HO^{\cdot} \quad (1)$$

$$HO^{\cdot} + H_2O_2 \rightarrow H_2O + HO_2^{\cdot} \quad (2)$$

$$c\text{–}Fe^{3+}\text{–}HO^{\cdot} + HO_2^{\cdot} \rightarrow H_2O + c\text{–}Fe^{3+} + {}^1O_2 \quad (3)$$

The homolytic scission of the H_2O_2 molecule, which is competitively inhibited by cyanide, includes a hydroxyl radical bound to the heme iron (c–Fe^{3+}) [2,16] [reaction (1)]; $HO^{\cdot}$ reacts with H_2O_2 with a rate constant of $3 \times 10^7\ M^{-1} \cdot s^{-1}$ [15] [reaction (2)], and reaction (3) accounts for singlet oxygen generation. The lack of effect of $HO^{\cdot}$ scavengers could be explained by the fact that the alcohol radicals are able to react with H_2O_2 to generate $HO^{\cdot}$ [17].

In this system, a superoxide dismutase activity by the Cu–amino acid chelates has been discarded by the lack of effect of the pure enzyme and the lack of correlation between affected chemiluminescence and superoxide dismutase activity of the Cu–amino acid complexes [18]. The catalase activity is also excluded since Cu^{2+} (free or as Cu–amino acid chelates) shows the same inhibitory effect on chemiluminescence whereas the catalase-like activity is only developed by Cu–amino acid complexes and not by the free Cu^{2+} itself [19]. The inhibition of chemiluminescence exerted by Cu^{2+} and Mn^{2+} might be due to either, (a) quenching of singlet oxygen by an external heavy atoms effect [20], or, (b) to effective scavenging of $HO^{\cdot}$. Since both cations did not affect much the Soret band bleaching, mediated by $HO^{\cdot}$, it seems likely that Cu^{2+} and Mn^{2+} are quenchers of singlet oxygen.

Acknowledgements

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