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Peroxidase-catalysed chlorophyll destruction accompanied by the formation of electronically excited states: chlorophyll emission not due to energy transfer from excited substrate metabolites [1]

Wilhelm J. Baader *, Virginia E. Gillo

Instituto de Química, Universidade de São Paulo, C.P. 26.077, 05599-970, São Paulo, S.P., Brazil

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Abstract

The peroxidase-catalysed oxidative degradation of chlorophyll-*a* (chl-*a*) in the absence or presence of 2,4-dichlorophenol (DCP) is accompanied by the generation of electronically excited states, as indicated by light emission in the red spectral region. A mechanism for the destruction of chl-*a* is proposed, which takes into account the formation of excited states. As peroxidative chl destruction is believed to be a naturally occurring degradation process, the formation of potentially deleterious excited states during degradation is of physiological importance. Furthermore, it is shown that the red light emission does not originate from energy transfer from a peroxidase substrate metabolite formed in the excited state. As chl-*a* has been widely utilized as a sensitizer for the detection of triplet excited species in various peroxidase-catalysed transformations in vitro and in vivo, the additional importance of this work is to show that the observation of red light emission in the presence of chl-*a* does not necessarily indicate the formation of triplet excited state substrate metabolites in peroxidase systems.

Keywords: Chlorophyll emission; Energy transfer; Peroxidase-catalysed chlorophyll destruction

1. Introduction

Despite the intense recent research in this field, the degradation of chlorophyll (chl) still appears to be a biological enigma [1]. The biochemical fate and products of chl disappearance are not yet clearly understood. In the natural environment, chl disappearance may be promoted by light, enzymes and microorganisms. The in vitro photo-oxidation of various chl derivatives is believed to involve singlet oxygen addition to the C(1)=C(20) double bond, following ring cleavage [2–10]. The initial photoproduct appears to be transformed in the dark to linear tetrapyrroles (bile pigments) [2]. The oxidative destruction of chl-*a* and pheophytin in micellar medium may occur with the involvement of the hydroxyl radical [9,10]. Recent work has also shown the photodegradation of the phytyl side-chain, with the involvement of oxygen free radicals, leading to the formation of acyclic isoprenoid compounds [11]. On the other hand, interaction of the superoxide anion with chl should lead to the rupture of the cyclopentenone ring V [12], in analogy with the well-known mechanism of allomerization [13–17].

Most of the chl degradation products in vitro and in vivo have not yet been completely identified [1,18–20]. Apart from chl derivatives with intact tetrapyrrole ring structures, low molecular weight organic acids [18,19], a pink pigment containing three major components and 13^2-hydroxychlorophyll have been identified [18–20]. More recently, several chl-*a* catabolites have been unambiguously identified. The catabolites, observed in different plant species, contain linear tetrapyrrole structures formed by ring opening at the C(4)–C(5) carbon bond [21].

Peroxidase-catalysed chl degradation has been reported to be dependent on hydrogen peroxide and monophenol. The enzyme utilized is commercial horseradish peroxidase or a thylakoid-bound enzyme isolated from plant tissues [22]. In addition, an oxygen-dependent chl oxidase, using fatty acids as cosubstrates, has been reported [19,23–25]. In this system, 13^2-hydroxychlorophyll has been identified as a reaction product [19]. This compound and chlorophyllin-*a*, a cyclopentenone V ring-opened derivative, are found in trace quantities in senescing sycamore leaves [24], which may indicate the involvement of the cyclopentenone V ring in the degradation process. However, subsequent studies have suggested

* Corresponding author. Tel.: +55 11 8183877; fax: +55 11 8155579.

[1] This work is dedicated to the late Prof. Dr. Giuseppe Cilento, whose sudden death appalled all of us who had known him as an outstanding scientist and an excellent human being.

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that 13^2-hydroxychlorophyll is merely a secondary breakdown product [25] arising from free radical allomerization [16]. Peroxidative chl degradation may be a naturally occurring breakdown process as both peroxidase and phenols are found within the chloroplast [1].

Apart from the research interest on its degradation, chl has been widely utilized as a sensitizer for the detection of electronically excited triplet species in the field of photochemistry and photobiology without light [26–33]. It has been shown that chl fluorescence can be elicited by energy transfer from enzymatically generated triplet carbonyls in various model systems [34–36]. Moreover, an elaborate photophysical study has shown that singlet excited chl can be formed by interaction of chl with triplet sensitizers [37]. Subsequently, chl has been frequently used for the detection of excited state formation in biochemical systems in vitro [38–41] and in vivo [42–45]. However, in some cases, extensive chl destruction has been observed [34,35] and, in some systems, chl-sensitized emission is the only indication of excited state formation [38,39,42,44].

In this work, we show that the peroxidase-catalysed destruction of chl-*a* in the absence and presence of 2,4-dichlorophenol (DCP) is accompanied by light emission, and we propose a mechanism for excited state formation. It is clearly shown that the observed emission in the presence of DCP is not due to energy transfer from excited state substrate metabolites to chl.

2. Experimental details

All chemicals were of analytical grade. Horseradish peroxidase (HRP) (type VI, EC 1.11.1.7, donor H_2O_2 oxidoreductase), chl-*a* and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, MO). Hexadecyltrimethylammonium bromide (CTAB), from Aldrich Chemical Company (Milwaukee, WI), was recrystallized from acetone–methanol (85 : 15), and DCP, obtained from Janssen Chemical Company, was sublimated (50 °C, 1 mmHg). The concentration of the stock solution of chl-*a* dissolved in ethanol was determined spectrophotometrically at 665 nm ($\varepsilon = 6.94 \times 10^4\ M^{-1}\ cm^{-1}$) [46]. The concentration of the HRP stock solution was determined from the absorbance at 403 nm ($\varepsilon = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$) [47]. The hydrogen peroxide concentration was determined by an iodometric assay using HRP as catalyst and photometric determination of $[I_3^-]$ [48]. DCP stock solutions were prepared by weight in ethanol.

Absorption spectra were recorded on a Zeiss DMR-10 spectrophotometer. Oxygen consumption was followed with a Yellow Springs Instruments model 53 oxygen monitor. Light emission was measured in counts per second ($c\ s^{-1}$) on a photon counter equipped with a red-sensitive Thorn EMI (model 9658AM) photomultiplier tube refrigerated by a Thorn EMI (model FACT 50MK III) thermoelectric cooler. The phototube output was connected to a Princeton Applied Research (model 1121A) amplifier–discriminator. The spectral distribution of the emission was obtained using a series of cut-off filters (Schott) covering the range 385–715 nm. Appropriate corrections were applied for the spectral characteristics of the filters and the response of the photomultiplier tube.

All experiments were performed at 35.0 ± 0.1 °C in aqueous 0.10 M phosphate buffer (pH 6.0) in a total volume of 3.00 ml, and were initiated by the addition of HRP stock solution. The final concentration of ethanol, used for the stock solutions of chl-*a* and DCP, was 0.1 M. The emission spectrum in Triton X-100 (Fig. 3, see later) was obtained by changing the cut-off filter during the relatively slow reaction, using the emission intensity without the filter as reference. In CTAB (Fig. 3, inset; Fig. 4, inset; see later), the spectra were obtained by performing independent experiments in triplicate for each filter and using the obtained maximum intensity in comparison with the maximum intensity without the filter. The experiments in anaerobiose were performed by incubation of the reaction mixture in the absence of HRP with glucose (1.16%) and glucose oxidase ($142\ mg\ ml^{-1}$) for 10 min at 35 °C in a cuvette closed with a septum. Control experiments indicated complete oxygen consumption under these conditions. The reaction was initiated by the addition of 10 μl of the HRP stock solution by means of a Hamilton syringe.

3. Results

In CTAB (1 mM) micelles, chl-*a* suffers destruction by the system DCP–H_2O_2–HRP, as indicated by the decrease in absorption at 665 nm (Fig. 1, inset). The spectrum obtained 5 min after the initiation of the reaction shows an almost complete disappearance of the 665 nm absorption, the appearance of an absorption around 520 nm, a considerable decrease in the 440 nm absorption (Soret region) and a strong increase

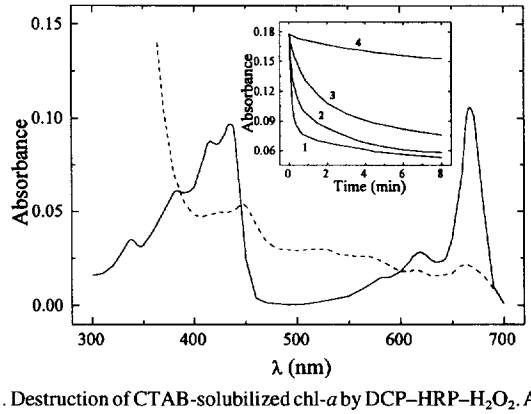


Fig. 1. Destruction of CTAB-solubilized chl-*a* by DCP–HRP–H_2O_2. Absorption spectra before (———) and after (– – –) exposure to peroxidase ([chl-*a*] = 1.5 μM, [DCP] = 0.20 mM, [H_2O_2] = 0.20 mM, [HRP] = 10 nM, [CTAB] = 1.0 mM). Inset: kinetics of chl-*a* destruction measured at 665 nm ([chl-*a*] = 2.8 μM, [DCP] = 0.20 mM, [H_2O_2] = 0.20 mM, [CTAB] = 1.0 mM; [HRP]: 1, 100 nM; 2, 10.0 nM; 3, 1.00 nM; 4, 0.00 nM).

in the absorption in the UV region. These spectral modifications are similar, although not identical, to those observed in chl destruction caused by superoxide anion [12] and the hydroxyl radical [9]. The destruction rate depends on [HRP]; in the absence of the enzyme, it is much lower (Fig. 1, inset). With 10 nM HRP, the rate of destruction shows a slight dependence on [DCP] (0.2–200 μM), but is independent of [H_2O_2] (20–200 μM) (data not shown). Similar results were obtained on substituting CTAB by Triton X-100 (0.25%).

Fig. 2 shows the observed emission kinetics from the system DCP–H_2O_2–HRP in CTAB micelles in the absence and presence of chl-*a*. Surprisingly, the system also shows strong emission in the absence of chl-*a*; however, this emission is not in the red spectral region. In the presence of chl-*a*, no drastic change is observed in the emission kinetics when the light emission is monitored over the whole spectral range. However, when using a 590 nm cut-off filter, rapid decay kinetics are obtained, concomitant with the kinetics of chl destruction under the same conditions (Fig. 1). No light emission is detected when using the same filter in the absence of chl-*a*.

The red emission observed in the presence of chl-*a* is independent of [DCP] and depends only very slightly on [H_2O_2]; however, the direct emission (in the absence of chl-*a*) is strongly dependent on [DCP] as well as on [H_2O_2] (Table 1). Therefore conditions can be found (at low [DCP] and low [H_2O_2]) where the direct emission is negligible and the red emission from chl-*a* cannot be generated by energy transfer from an excited state DCP metabolite. Instead, this emission must be related to the degradation reaction of chl-*a*. The decay rate of light emission and the maximum emission intensity increase with [HRP] in parallel with the behaviour of chl-*a* destruction kinetics.

The emission spectra, obtained with the aid of cut-off filters, for the system in the presence of chl-*a* in CTAB and Triton X-100 micelles largely match the fluorescence spectrum of chl-*a* [34–36,49], except for a shoulder at approximately 625 nm observed in CTAB micelles (Fig. 3). An

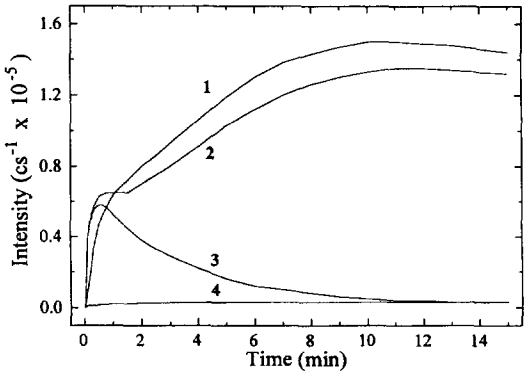


Fig. 2. Time course of the emission from the system DCP(0.20 mM)–HRP(1.0 nM)–H_2O_2(0.20 mM) in the absence and presence of chl-*a* (1.0 μM) and in the absence and presence of a red filter (590 nm): 1, DCP–HRP–H_2O_2; 2, DCP–HRP–H_2O_2–chl-*a*; 3, DCP–HRP–H_2O_2–chl-*a*–cut-off filter 590 nm; 4, DCP–HRP–H_2O_2–cut-off filter 590 nm.

Table 1
Comparison of the maximum emission intensity from the system DCP–H_2O_2–HRP in the absence and presence of chl-*a*[a]

DCP (μM)	H_2O_2 (μM)	Chl-*a* (μM)	I_{max} (10^3 c s^{-1})	t_{max} (min)
200	200	–	18.0	0.5
200	200	1.0	17.5[b]	0.1
20	200	–	1.60	2.8
20	200	1.0	18.0[b]	0.1
20	20	–	0.5	2.0
20	20	1.0	12.0[b]	0.1

[a][CTAB] = 1.0 mM; [HRP] = 10 nM. [b]In the presence of a 610 nm cut-off filter.

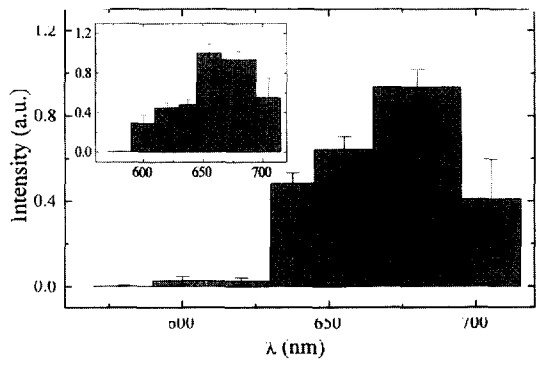


Fig. 3. Emission spectra from the system DCP–H_2O_2–HRP–chl-*a* in Triton X-100 and CTAB (inset) micelles ([Triton X-100] = 0.25%, [chl-*a*] = 3.5 μM, [DCP] = 0.20 mM, [H_2O_2] = 0.20 mM, [HRP] = 10 nM; inset: [CTAB] = 1.0 mM, [chl-*a*] = 2.0 μM, [DCP] = 20 μM, [H_2O_2] = 20 μM, [HRP] = 10 nM).

additional chl-*a* emission in this spectral region (600–625 nm) has been observed in some peroxidase systems (believed to generate triplet carbonyls) in which chl-*a* has been utilized as a triplet energy acceptor [34–36]. Interestingly, a band at 625 nm has been attributed to chl-*a* dimer emission [50], and a weak 628 nm emission band has been shown to originate from chl adsorbed to polyethylene particles [51]. However, the origin of the additional emission band in our system cannot be due to the above possibilities, as the fluorescence spectrum obtained by optical excitation at 418 nm does not show the additional emission band (data not shown).

Finally, some experiments were performed on chl-*a* destruction in the absence of DCP. In the system chl-*a*–H_2O_2–HRP in CTAB micelles, chl destruction is observed, which is accompanied by light emission, although the kinetics are considerably slower and the emission intensity at least ten times lower (Fig. 4) than that observed in the same conditions in the presence of DCP (20 μM). The emission and chl-*a* destruction are totally abolished under complete anaerobiosis (treatment of the reaction medium with glucose/glucose oxidase before the initiation of the reaction by the addition of HRP) (Fig. 4). It should be noted that no measurable oxygen consumption was observed in all the experimental conditions used.

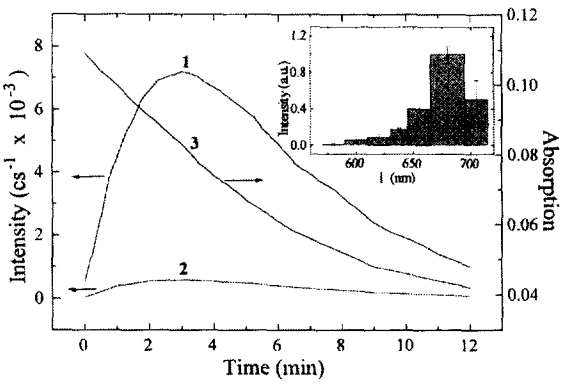


Fig. 4. Time course of chl-*a* destruction, light emission kinetics and spectral distribution of the emission from the system chl-*a* (5.0 μM)–H_2O_2 (20 μM)–HRP (10 nM) in CTAB (1.0 mM) micelles: 1, emission under aerobiose; 2, emission under anaerobiose (treatment with glucose (1.16%) and glucose oxidase (142 mg ml^{-1}) for 7 min before addition of HRP); 3, absorption at 665 nm. Inset: emission spectrum (see Section 2).

4. Discussion

Our experimental results clearly indicate that the oxidative peroxidase-catalysed destruction of chl is accompanied by light emission in the red spectral region. In the presence of DCP, a bright emission, spectrally similar to chl-*a* fluorescence, is observed. Although the system presents emission in the absence of chl-*a*, conditions can be found where it is extremely weak. The strong red emission, observed in these conditions in the presence of chl-*a*, must be due to the chl-*a* destruction reaction, and is not caused by energy transfer from a possible chemiexcited species formed by metabolization of DCP by HRP–H_2O_2.

The main role of DCP in the emissive chl-*a* destruction reaction is as a cosubstrate which, as a good hydrogen donor, rapidly reacts with peroxidase compound II (HRP-II). As the reaction of HRP-II, which is a much weaker oxidant than HRP-I, with chl-*a* is slow, the fast reaction of DCP with HRP-II leads to an acceleration of chl-*a* degradation due to fast conversion of unreactive HRP-II to the native peroxidase (HRP). The whole reaction sequence is expected to involve a conventional peroxidase/oxidase cycle [52] (Scheme 1).

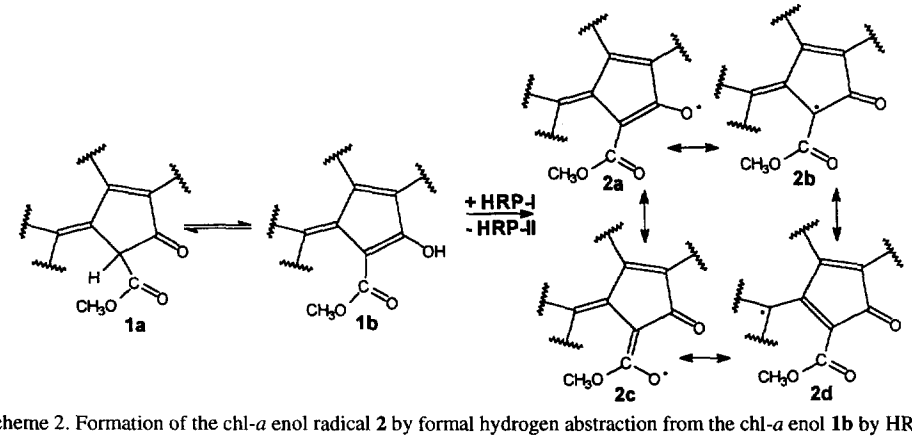
The above scheme also readily explains the observed slower chl-*a* destruction in the absence of DCP, which is also accompanied by red light emission. Although the emission intensity in this case is much lower, these experiments unequivocally show the possibility of observing light emission in the peroxidase–H_2O_2–chl-*a* system in the absence of triplet carbonyl species not related to the chl destruction reaction.

The reaction of chl-*a* with HRP-I is expected to proceed by a formal hydrogen abstraction from the enol form **1b** of the chl-*a* cyclopentenone ring V [16,17], in analogy with the well-characterized reaction of 2-methylpropanal with peroxidase/oxidase [53]. The initiation of chl-*a* degradation on cyclopentenone ring V has been proposed by several workers [12–17]. The formal enol radical generated is highly resonance stabilized; the unpaired electron is delocalized through the porphyrin ring as indicated by resonance structure **2d** (Scheme 2).

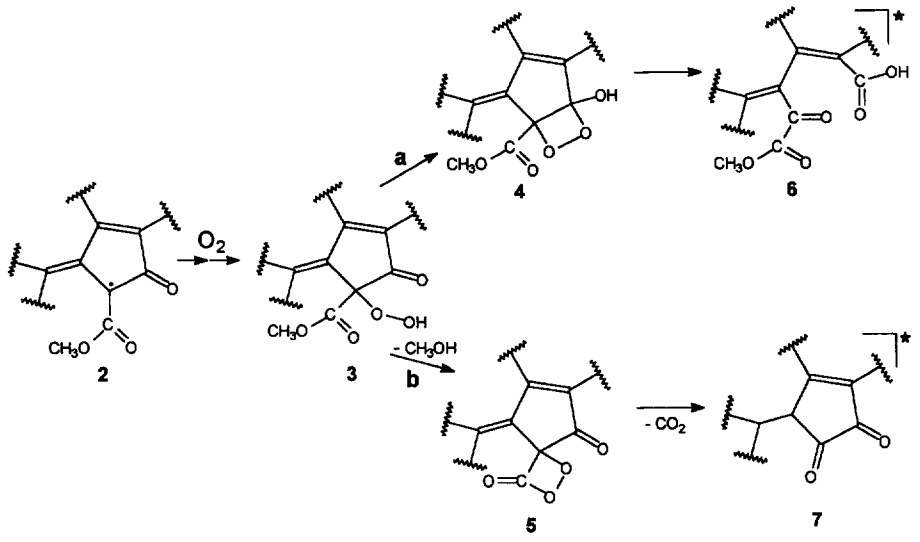
The reaction of free radical **2** with oxygen leads to a peroxyradical which is transformed to the hydroperoxide **3** (Scheme 3), in analogy with several well-studied chemiluminescent peroxidase/oxidase reactions [26–33,53]. The importance of oxygen for the emissive chl-*a* destruction is demonstrated by the experiments under anaerobiosis (Fig. 4). The fact that no oxygen consumption could be observed is not surprising as chl-*a* is used only in micromolar quantities and, assuming a 1 : 1 stoichiometry, only about 1% of the dissolved oxygen ($[O_2] \sim 2 \times 10^{-4}$ M) [54] is consumed by the reaction with chl-*a*. This value is considerably below the resolution of our instrument. The hydroperoxide **3** can cyclize by nucleophilic hydroperoxy attack on the car-

$$\text{HRP} + H_2O_2 \xrightarrow{\text{fast}} \text{HRP-I} + H_2O$$
$$\text{HRP-I} + \text{chl-a} \xrightarrow{\text{fast}} \text{HRP-II} + \text{chl-a}^{\cdot}$$
$$\text{HRP-I} + \text{ArOH} \xrightarrow{\text{fast}} \text{HRP-II} + \text{ArO}^{\cdot}$$
$$\text{HRP-II} + \text{chl-a} \xrightarrow{\text{slow}} \text{HRP} + \text{chl-a}^{\cdot}$$
$$\text{HRP-II} + \text{ArOH} \xrightarrow{\text{fast}} \text{HRP} + \text{ArO}^{\cdot}$$
$$\text{chl-a}^{\cdot} + O_2 \longrightarrow \text{products} + h\nu$$

Scheme 1. Conventional peroxidase/oxidase cycle [52] for the co-oxidation of DCP and chl-*a*.



Scheme 2. Formation of the chl-*a* enol radical **2** by formal hydrogen abstraction from the chl-*a* enol **1b** by HRP-I.



Scheme 3. Proposed sequence for the peroxidase-catalysed chl-*a* destruction by reaction of the enol radical **2** with oxygen and formation of the cleavage products **6** and **7**.

bonyl or ester moiety, giving the 1,2-dioxetane **4** or the 1,2-dioxetanone (α-peroxylactone) **5** respectively. The four-membered ring peroxides suffer cleavage, forming the products **6** and **7** respectively (see below). As it is well known that these cyclic peroxides furnish excited state cleavage products [26–33], the reaction sequence outlined in Scheme 3 readily explains the observed light emission during chl degradation.

The proposed path a is closely related to the suggested mechanism of chl allomerization and degradation on aging of plants [12–17]. Most importantly, the hydroperoxide **3** and the dioxetane decomposition product **6** have already been proposed as intermediates in the allomerization of chl [16,17].

The nature of the emissive species cannot be unequivocally identified from our experimental data. The similarity of the chemiluminescence spectrum to the fluorescence spectrum of chl-*a* indicates that unreacted chl-*a* itself, or a species with similar emission properties, should be responsible for the light emission. On the basis of Scheme 3, several possibilities for the origin of the light emission exist: (1) the unimolecular decomposition of the dioxetane **4** and the dioxetanone **5** is expected to lead to excited state generation, although triplet states, commonly showing low emission quantum yields, should be preferentially formed [26]; (2) the above-mentioned triplet excited species can transfer their excitation energy to unreacted chl-*a* (via triplet–singlet energy transfer) eliciting chl fluorescence [37]; (3) the dioxetanone **5**, but not the dioxetane **4** [26,55–57], may interact with unreacted chl-*a* by an intermolecular chemically induced electron exchange luminescence (CIEEL) process [55], leading to the decomposition product **7** and singlet excited chl-*a* and, consequently, to chl-*a* fluorescence emission; it is known that chl-*a* is a very efficient activator of the CIEEL process with appropriate peroxides [58]; (4) the dioxetanone **5**, and also the dioxetane **4**, may suffer decomposition by an intramolecular CIEEL process, where the porphyrin-magnesium moiety of the appropriate compound serves as the electron donor initiating the sequence [55]; this intramolecular CIEEL process is expected to lead to the singlet excited state of the porphyrin-magnesium moiety, which should show an emission spectrum very similar to the fluorescence spectrum of unaltered chl-*a*; it is known that 1,2-dioxetanes containing easily oxidizable substituents decompose by an intramolecular CIEEL mechanism, with efficient generation of singlet excited states [59].

Although, from our experimental data, we cannot distinguish between the abovementioned possibilities, we believe that the latter is the most probable due to our knowledge about the properties of four-membered ring peroxides.

5. Conclusions

Chl degradation by HRP–H_2O_2 is accompanied by light emission, arising from an excited state formed during the course of the chl-*a* degradation sequence. The presence of DCP as cosubstrate leads to an acceleration of the degradation reaction and to an increase in the light emission intensity. The excited state generation is assumed to proceed by the cleavage of the four-membered ring peroxides **4** and/or **5** (Scheme 3) by an intramolecular CIEEL mechanism. The observation of excited state generation is important on its own, as peroxidase-catalysed chl destruction may be a naturally occurring degradation process [1] and excited states may have deleterious effects [26–33].

Furthermore, it was shown that, in certain conditions, light emission is not due to energy transfer from triplet excited cosubstrate metabolites, but instead is directly linked to chl-*a* destruction. As chl-*a* is frequently utilized as a sensitizer for the detection of enzymatically generated triplet excited carbonyl species in vitro and in vivo, [26–45], an additional

importance of this work was to show that the observation of red light emission in the presence of chl-*a* cannot necessarily be taken as proof of the formation of triplet excited substrate metabolites during peroxidase-catalysed reactions.

A similar observation has been made in the peroxidase-catalysed indol-3-acetic acid oxidation in the presence of xanthene dyes, where it was shown that the observed dye emission was due to the co-oxidation of xanthene dyes [60]. It should be stated, however, that several well-characterized peroxidase-catalysed reactions exist which undoubtedly lead to the formation of triplet excited carbonyl compounds [26–33,53], and where the sensitized emission in the presence of chl and xanthene dyes should be due to energy transfer from the triplet excited species to the sensitizer dyes. It is well documented that chl-*a* can act as an efficient triplet energy acceptor, a process which leads to chl fluorescence [37]. On the other hand, in several complex systems, where the observation of chl-*a* fluorescence is the only evidence for the occurrence of triplet excited species, this may not necessarily be true. The observed emission, at least in some cases where destruction of chl-*a* has been verified, may be related to the chl destruction reaction and not to energy transfer from triplet excited metabolites.

Acknowledgements

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