OXIDATION PHOTOSENSITIZED BY 2-CHLOROTHIOXANTHONE: THE ROLE OF SINGLET OXYGEN†

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Summary

2-chlorothioxanthone, solubilized in sodium dodecylsulphate micelles, photosensitized the oxidation of methionine. The reaction was quenched by azide ion and 1,4-diazabicyclo[2.2.2]octane in accordance with a mechanism involving $O_2(^1\Delta_g)$. 2-chlorothioxanthone was incorporated in the bilayer membrane of lipid vesicles where it performed a dual role of photosensitizer and fluorescent probe. The rate of photosensitized oxidation increased with increasing egg phosphatidylcholine in the membrane. The process was quenched efficiently by azide ion showing that $O_2(^1\Delta_g)$ plays an important part. On irradiation of a mixture of dipalmitoyl-L-α-phosphatidylcholine vesicles containing membrane-bound chlorothioxanthone and egg phosphatidylcholine vesicles containing no chlorothioxanthone, oxygen absorption occurred. It is concluded that $O_2(^1\Delta_g)$ diffused from its site of formation in the membranes of the dipalmitoylphosphatidylcholine vesicles, through the intervening aqueous phase, into the egg phosphatidylcholine vesicles where it oxidized the unsaturated fatty acid side-chains.

1. Introduction

The photosensitized oxidation of model membrane systems is of interest in connection with the mechanism of oxidative damage to cell membranes which leads to lysis [1]. For example, protoporphyrin, present in the red blood cells of patients with the genetic disorder erythropoietic protoporphyria, is an efficient photosensitizer and causes photohaemolysis [2]. There is growing interest, too, in the use of photosensitization for the treatment of certain forms of cancer. In studies of photosensitized oxidation of membrane systems the site of action of the sensitizer is an important consideration, since it may influence both the mechanism and the efficiency of the process. One reason for our choice of 2-chlorothioxanthone (CTX) I

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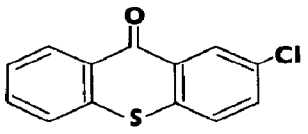

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as a photosensitizer, therefore, was its extremely low solubility in water and its appreciable solubility in organic solvents. This ensures that in model membrane systems, such as lipid vesicles, the CTX is located almost exclusively in the lipid bilayer.

CTX is well known to photosensitize reactions by a type I (radical) pathway and is an important sensitizer in the photocuring industry [3]. In this application CTX is strongly inhibited by oxygen, which suggests that formation of $O_2(^1\Delta_g)$ may be an important pathway for deactivation of the excited triplet state. We were interested to discover whether oxidation of biochemical systems, sensitized by CTX, would be governed by a type I or a type II (in particular an $O_2(^1\Delta_g)$) pathway.

Another feature of CTX which interested us is the marked dependence of its fluorescence intensity on solvent polarity. In cyclohexane its fluorescence yield is extremely low (very much less than 0.01). Its fluorescence increases dramatically with increasing solvent polarity. By analogy with results for thioxanthone [4] the first excited singlet state of CTX is π,π^*. The dependence of thioxanthone fluorescence (and by inference that of CTX) on solvent polarity appears to be due to a proximity effect involving the $S_2(n,\pi^*)$ and $S_1(\pi,\pi^*)$ states [5]. The electronic energy gap between these states is greater when CTX is present in polar solvents than when it is present in non-polar solvents; in polar solvents this favours fluorescence over S_1–S_0 internal conversion. In non-polar media, the proximity of the n,π^* and π,π^* states favours internal conversion to the ground state, producing a corresponding fall in fluorescence intensity [6]. This property made CTX a useful fluorescent probe in this investigation; we discovered later that the possible use of thioxanthone as a fluorescent probe had been suggested previously by Dalton and Montgomery [4].

2. Experimental details

An oxygen electrode (Rank Brothers, Cambridge) was employed to measure the oxygen absorption in reactions photosensitized by CTX. The oxygen electrode had a borosilicate glass cell and the temperature was regulated (usually at 25 °C) by circulating water through the jacket by means of a Gallenkamp water circulator (type TM 920). Oxygen, present in solutions, equilibrated across a Teflon membrane (25 μm thick) and was reduced at the negatively polarized smooth platinum electrode. The small current generated was amplified and the signal was displayed on a Servoscribe linear recorder, the response of which was proportional to the concentration of

oxygen in the solution under investigation. The solution (3 cm^3), which was continuously stirred, was irradiated with light from a microscope illuminator (Olympus) having a 30 W tungsten filament bulb. The distance from the irradiation source to the centre of the oxygen electrode cell was 200 mm. The rates of oxygen absorption were calculated using the expression

$$\text{rate} = \frac{cS}{x}\ \mu\text{mol dm}^{-3}\ \text{s}^{-1}$$

where c (μmol dm^{-3}) is the concentration of oxygen in the air-saturated solution at 25 °C, S (mV s^{-1}) is the slope of the recorder trace and x (mV) is the total recorder deflection on adding sodium dithionite to the air-saturated solution. For the air-saturated aqueous solution [7] c is 237 μmol dm^{-3}.

CTX was incorporated in the membranes of lipid vesicles which were prepared by the method of Kremer *et al.* [8]. The lipids, dipalmitoyl-L-α-phosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC) and egg phosphatidylcholine (PC) (Sigma Chemical Company) were made up in chloroform solution. Appropriate volumes of these solutions were measured out and the chloroform was evaporated under a stream of nitrogen. An ethanol solution of CTX (1×10^{-3} M, 60 μl) was added and the lipids were dissolved. The resulting solution was injected, at a controlled rate, from a 100 μl syringe into 0.1 M phosphate buffer (pH 7.0; 6 cm^3) at 60 °C, while the buffer was vortexed. The resulting vesicle suspension was incubated under nitrogen-saturated conditions at 60 °C for 30 min. In each experiment, conducted in duplicate, the vesicle suspension (3 cm^3) was aerated and irradiated in the oxygen electrode.

3. Results and discussion

On increasing the polarity of the solvent, the longest wavelength absorption band of CTX showed a red shift (Fig. 1), consistent with this band being $\pi \rightarrow \pi^*$ in character. CTX was solubilized by warming the solid with 1 wt.% aqueous sodium dodecylsulphate (SDS) solution. The maximum of the longest wavelength absorption band of CTX in SDS is red shifted compared with that in cyclohexane. The band displays none of the structure observed in the latter solvent (Fig. 1). This indicates that the CTX is located near the periphery of the micelles rather than being buried in the non-polar interior. This is confirmed by the high fluorescence intensity of CTX in SDS micelles, which indicates a strong interaction with the aqueous phase.

CTX in SDS micelles photosensitized the oxidation of methionine. Oxygen absorption was quenched efficiently by azide ion; the quenching followed Stern–Volmer kinetics with a quenching constant of $4 \times 10^3\ M^{-1}$. Oxygen absorption was halted completely if sufficient azide ion was present. When the rate constant for reaction of $O_2(^1\Delta_g)$ with methionine [9] (3×10^7 $M^{-1}\ s^{-1}$), the rate constant for quenching of $O_2(^1\Delta_g)$ by azide ion [10]

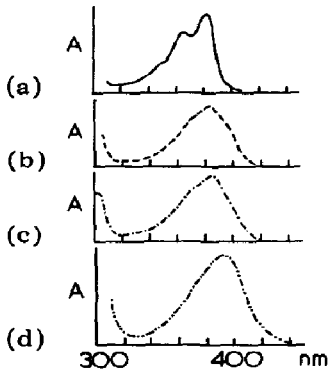


Fig. 1. Dependence of the absorption spectrum of CTX on the solvent: (a) cyclohexane; (b) *n*-octanol; (c) ethanol; (d) sodium dodecylsulphate (SDS). (The broad red-shifted spectrum of CTX in SDS micelles should be noted.)

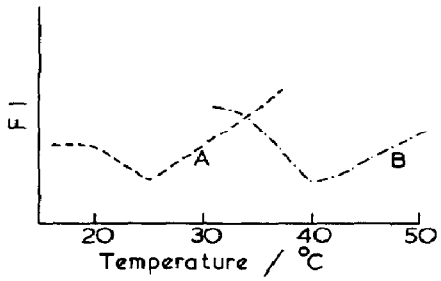


Fig. 2. Influence of temperature on the fluorescence of CTX incorporated in the membranes of DMPC (curve A) and DPPC (curve B) vesicles (FI, fluorescence intensity).

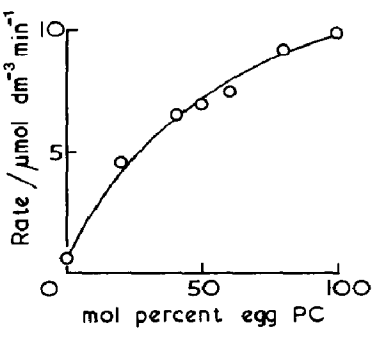
($2 \times 10^9\ M^{-1}\ s^{-1}$) and the concentration of methionine (2×10^{-2} M) are taken into consideration, the slope of the Stern–Volmer plot is consistent with simple competition kinetics between azide and methionine for $O_2(^1\Delta_g)$.

The CTX-photosensitized oxidation of methionine was quenched also by 1,4-diazabicyclo[2.2.2]octane. The Stern–Volmer quenching constant was $1.5 \times 10^2\ M^{-1}$ compared with $4 \times 10^3\ M^{-1}$ for the more efficient quencher azide ion.

Figure 2 shows the fluorescence changes observed on heating vesicles of DPPC and of DMPC in which CTX was incorporated in the bilayer. The heating rate was 1 °C min^{-1}. With increasing temperature, the fluorescence of CTX first fell then rose again. The minima correspond closely to the known transition temperatures between the gel and the liquid crystalline states of the bilayer of the respective vesicles. Almost exactly the same curves were obtained on cooling, very little hysteresis being observed. The increase in fluorescence beyond the transition temperature indicates an increasing interaction of the CTX with water molecules.

Figure 3 shows the dependence of the rate of oxygen absorption on the mole fraction of egg PC in the DPPC–(egg PC) vesicle membranes. The rate increases with increasing unsaturation of the fatty acid side-chains of the lecithins.

The effect of added azide ion on the CTX-photosensitized oxidation of egg PC vesicles is shown in Fig. 4. The quenching is efficient, the quenching constant being $1.1 \times 10^3\ M^{-1}$. This shows that $O_2(^1\Delta_g)$ plays a major role in oxidation photosensitized by CTX, although this does not preclude type I processes. Azide ion was about 7% more efficient when added to the buffer before injection of the lipid solution rather than afterwards. The presence of azide ion in the aqueous phase inside the vesicles, as well as outside, probably explains this greater quenching efficiency.



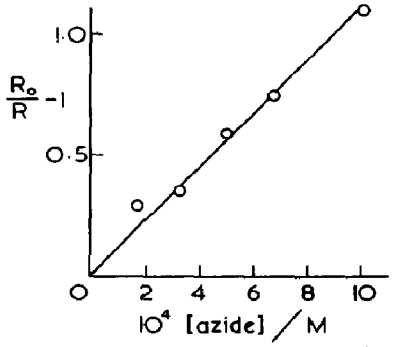


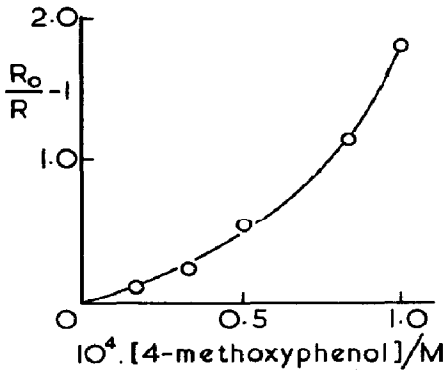
Fig. 3. Dependence of the rate of CTX-photosensitized oxygen absorption on the mole percentage of egg PC in DPPC–(egg PC) vesicle membranes.

Fig. 4. Quenching, by azide ion, of the CTX-photosensitized oxidation of egg PC vesicles (R_0, rate of oxygen absorption in the absence of azide ion; R, rate obtained at various azide ion concentrations).

As Kraljic *et al.* [11] have pointed out, azide is very polar and is expected to be insoluble in the hydrophobic interior of the bilayer. It is probable that quenching of $O_2(^1\Delta_g)$ takes place in the aqueous phase outside the bilayer.

The effect of 4-methoxyphenol (PMP) (as a potential radical scavenger) on CTX-photosensitized oxidation of egg PC vesicles was investigated. The result (Fig. 5) shows that PMP is an efficient quencher of the photosensitized oxidation, although the curved plot indicates a more complex protective effect than that observed with azide ion. If the PMP was added to the phosphate buffer before injection of the CTX–(egg PC) solution, the quenching was about 18% more efficient. In partition experiments with water and *n*-octanol, 89% of the PMP partitioned into the organic phase. Thus PMP would be expected to be present both in the bilayers of the vesicles and in the aqueous phase. The PMP effect is difficult to interpret. It appears to indicate a type I component or possibly a radical mechanism involving the breakdown of hydroperoxides. However, certain phenols are known to quench singlet oxygen, some with high efficiency [9]. The effect of PMP on the CTX-photosensitized oxidation of methionine was investigated therefore. For a 2×10^{-3} M solution of methionine the rate of oxygen absorption was halved by 1×10^{-3} M PMP, possibly as a result of $O_2(^1\Delta_g)$ quenching. Clearly, the effect of PMP is complex and warrants further investigation.

In further experiments CTX was incorporated in the membranes of DPPC vesicles. An aliquot of this vesicle suspension was mixed with an equal volume of egg PC vesicles which contained no sensitizer. The rate of photosensitized oxidation was measured as a function of incubation (25 °C) time. The result is shown in Fig. 6. The shortest possible incubation time was 2 min, because of the necessity to aerate the vesicle suspension and to obtain a steady recorder trace before illumination. As can be seen from



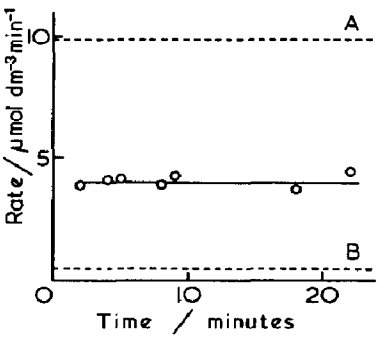


Fig. 5. Quenching, by PMP, of the CTX-photosensitized oxidation of egg PC vesicles (R_0, rate of oxygen absorption in the absence of PMP; R, rate obtained at various PMP concentrations).

Fig. 6. Rate of CTX-photosensitized oxidation as a function of incubation time following the mixing of DPPC vesicles, having membrane-bound CTX, with egg PC vesicles having no CTX (—○—): line A, rate for all-egg PC vesicles; line B, rate for all-DPPC vesicles.

Fig. 6 the rate was independent of the contact time between the two types of vesicles. The rate was approximately 60% of that obtained for vesicles in which equimolar quantities of DPPC and egg PC were present, together with CTX in the *same* bilayer. We interpret this result in terms of the diffusion of $O_2(^1\Delta_g)$ from the site of formation in the DPPC vesicles into the egg PC vesicles where it oxidized the double bonds, some 40% of the $O_2(^1\Delta_g)$ apparently being deactivated before reaching its target.

The question of whether CTX undergoes intervesicular exchange is important; if it does, the process must occur in less than 2 min, otherwise an increase in rate with incubation time would be expected. Analogous results have been obtained by Rodgers and Bates [12] who incorporated a sensitizer in the membranes of one set of vesicles and diphenylisobenzofuran (DPBF) in another set. Oxidation of the DPBF occurred when a mixture of the two sets of vesicles was irradiated. They interpreted this in terms of the diffusion of $O_2(^1\Delta_g)$ from its site of formation into the bilayer of a different vesicle where oxidation of DPBF occurred. They argued, from work by Uster and Deamer [13], that lipid-soluble probes in liposomal structures will not undergo exchange between liposomes unless a fusion-inducing additive is present. We were able to use the properties of CTX as a fluorescent probe to test the possibility of fusion between different vesicles. CTX was incorporated in DPPC and in DMPC vesicles. Equal volumes of the two vesicle suspensions were mixed and the fluorescence of CTX was monitored as a function of temperature. The fluorescence–temperature profile on heating (1 °C min^{-1}) (Fig. 7) showed two minima corresponding to the transition temperatures of DMPC and DPPC (*cf.* Fig. 2). However, when the vesicle mixture was taken to 60 °C and then cooled, only one minimum was observed. This single minimum was also

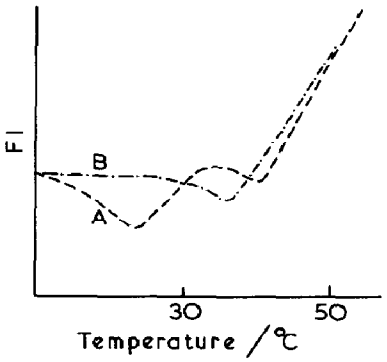


Fig. 7. Fluorescence changes of CTX on heating (curve A) and cooling (curve B) a mixture of DPPC and DMPC vesicles, both having membrane-bound CTX (FI, fluorescence intensity).

seen on increasing the temperature, but only if the mixed vesicles had been pre-incubated at 60 °C beforehand.

Vesicles made by injection of equimolar quantities of DMPC and DPPC together with CTX into phosphate buffer also showed the single minimum. These experiments show that vesicle fusion does not occur at the temperature of the photo-oxidation experiments, but it does occur at higher temperatures (about 60 °C). It is assumed that this also holds for vesicles composed of egg PC, although this was not tested because egg PC vesicles do not show a phase transition in a convenient temperature range (and correspondingly they do not show a minimum in the CTX fluorescence–temperature profile).

Rodgers and Bates [12] obtained the same rate of DPBF bleaching when the sensitizer was located in the bilayers of separate vesicles as was obtained when both the sensitizer and the DPBF were cosolubilized in the bilayers of the same vesicles. Although, in our experiments, a lower rate was obtained when the sensitizer and oxidizable substrate were in separate vesicles, the observations lead us to a similar conclusion, *i.e.* that $O_2(^1\Delta_g)$ diffuses from its site of formation (a vesicle containing CTX) through the intervening aqueous phase and into an egg PC vesicle membrane where it oxidizes the unsaturated fatty acid side-chains. This diffusion of $O_2(^1\Delta_g)$ in and out of vesicles also explains why azide ion, although present in the aqueous phase, quenches the oxidation of egg PC vesicles with high efficiency, even though the photosensitizer is solubilized in the lipid bilayers.

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