Sensitization Mechanism in the Fluorescein Photosensitized Reduction of Ferric Ion by EDTA*

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The primary steps in the photoreduction of ferric ion by EDTA using fluorescein as sensitizer (aqueous solution, pH 5.5) were studied by means of the flash photolysis technique. The results show that the dye in the triplet state is oxidized by ferric chelate to the semioxidized dye radical. There is strong indication that two semioxidized dye radicals react with each other to give a dimer which in turn is reduced rapidly by free EDTA regenerating the dye in its original state. It is also shown that the triplet is reduced by EDTA to the semireduced radical; however, in the photosensitized reaction this reduction is not important compared to the very efficient photo-oxidation process. Rate constants for the different reaction steps were determined.

Previous studies have shown that fluorescein [1,2] and eosin [3] are easily photo-oxidized to the corresponding semioxidized dye radicals in aqueous solution in the presence of oxidizing agents. Under appropriate conditions a fast reverse reaction regenerates the dye in its original state and no permanent photochemical change is observed. It was suggested [2] on basis of this behaviour that the semioxidized dye might in certain cases function as an intermediate in reduction-oxidation reactions photosensitized by fluorescein dyes. The energy absorbed by the dye would in this case be transferred to the reactants in a cycle of chemical processes: reaction of the photoexcited dye with the oxidizing component to give the semioxidized dye, followed by the reduction of the latter species by the reducing component regenerating the dye. The present paper reports an investigation of the possible occurrence of this type of photosensitization mechanism in the photosensitized reduction of ferric ion by ethylene diamine tetraacetic acid (EDTA) in the presence of fluorescein as sensitizer. Oster and Oster [4] showed that this is an efficient photochemical reaction (which can be induced by a variety of dyes), and they proposed a reaction mechanism based on quantum yield measurements. In the present

study the flash photolysis method was used; the appearance and disappearance of short-lived intermediary products were measured directly thus allowing a detailed picture of the mechanism to be obtained.

EXPERIMENTAL

Solutions were prepared from chromatographically pure fluorescein, the disodium salt of EDTA, ferric sulfate, and triple-distilled water buffered at pH 5.5 (0.02 M acetic acid, 0.13 M sodium acetate). Reagents were analytical grade. The solutions were degassed thoroughly by repeated evacuation and alternate saturation with oxygen-free argon.[2]

The flash photolysis apparatus has been described in detail.[1,5] Discharge energy was 6 kJ and flash duration time 30 μsec. The solutions were contained in cylindrical silica cells (60 cm long, i.d. 1.2 cm) with plane end windows. A surrounding jacket was filled with a ferric alum solution which absorbed all light below 380 mμ. Changes in light transmission induced by the flash were measured photoelectrically at selected wavelengths as a function of time using oscilloscopic recording.

RESULTS

The fluorescein photosensitized reduction of ferric ion by EDTA is a complex reaction involving several intermediary products. To facilitate the kinetic analysis comparative studies were done of solutions containing 1) the dye alone, 2) dye and EDTA, and 3) the complete reaction system. Excitation was limited to the visible, where only the photosensitizer is absorbing; absorption maxima of the dye are at 455 and 475 mμ. The photoexcited dye may return to the ground state by fluorescence or radiationless deactivation, or may cross over to the triplet state; direct photochemical decomposition of the dye does not occur. Since the concentrations of the reactants of the photosensitized reaction were kept low, and since there was no indication of association between the dye and the reactants (as judged from absorption spectra), direct chemical reaction of the dye in the short-lived fluorescent state was not expected to be of any importance. Under these conditions the dye reacts *via* its triplet state which is accessible to study by the flash photolysis technique. In the present study the flashing conditions were chosen such as to produce quantitative conversion of the dye to the triplet state, and a low concentration of dye was used (0.1 μM) in order to suppress concentration quenching of the triplet dye.

Fluorescein in the absence of reactants. It is known from previous studies[1] that the triplet dye disappears according to the following scheme in the absence of quenching agents:

$$T \rightarrow S \tag{1}$$

$$2\,T \rightarrow T + S \tag{2}$$

$$T + S \rightarrow 2\,S \tag{3}$$

$$2\,T \rightarrow X + R \tag{4}$$

$$T + S \rightarrow X + R \tag{5}$$

where S, T, X, and R denote the dye in the ground state, in the lowest triplet state, in a semioxidized state, and in a semireduced state, respectively.
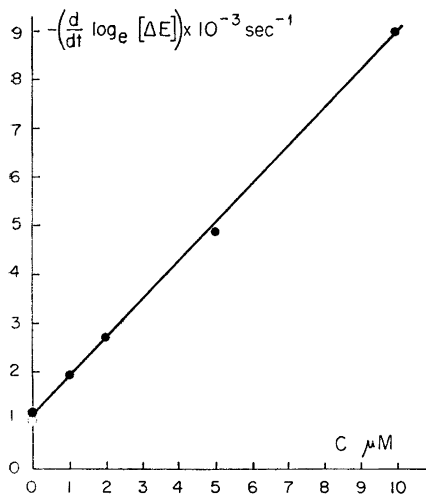
Fig. 1. Rate of decay of the transient optical density (ΔE) at 520 mμ at the initial stage of decay, after flashing 0.1 μM fluorescein in the presence of 1 mM EDTA and ferric chelate of concentration C μM (●). The triplet concentration is proportional to ΔE at this wavelength. For comparison the corresponding rate observed in the absence of reactants is also shown (○).
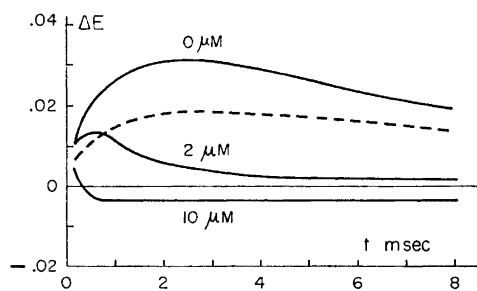
Fig. 2. Formation of semireduced fluorescein: transient change in optical density observed at 355 mμ after flashing 0.1 μM fluorescein, – – – – in the absence of reactants, and ——— in the presence of 1 mM EDTA and ferric chelate of concentration indicated in the figure. Time t refers to the time after the decay of the flash. Cell length is 60 cm.

Flash excitation of a 0.1 μM fluorescein solution produced a transient absorption at 520 mμ due to conversion of the dye to the triplet state which absorbs strongly ($\varepsilon = 5 \times 10^3$) at this wavelength.[1] (S, X, and R do not absorb at 520 mμ.) The triplet decayed approximately exponentially after the flash, with a first-order rate constant of 1.00×10^3 sec^{-1} in the stage immediately following the flash (Fig. 1).

The appearance of semireduced fluorescein was measured at 355 mμ where this radical has a pronounced absorption peak[1] ($\varepsilon = 3 \times 10^4$); the results are shown in Fig. 2. Other species present in the solution absorb only weakly at this wavelength. The increase in absorption during the flash is due to the triplet dye which has a higher extinction coefficient at 355 mμ than the ground-state dye. The further increase after the flash is due to semireduced dye formed by reactions (4) and (5). The semioxidized dye appeared simultaneously, and was studied at its absorption peak at 428 mμ[1] ($\varepsilon = 5 \times 10^4$). At this wavelength the dye itself absorbs strongly ($\varepsilon = 1.8 \times 10^4$). Fig. 3 shows the decrease in optical density at 428 mμ during the flash due to the conversion of the strongly absorbing fluorescein to the weakly absorbing triplet state; the enhanced optical density at the later stage of the reaction results from the formation of semioxidized dye by reactions (4) and (5). The slow decay of the
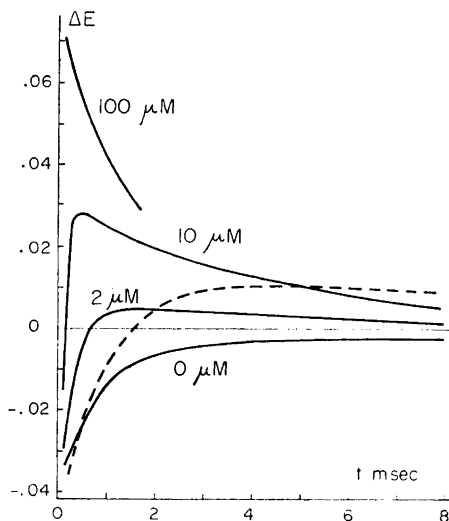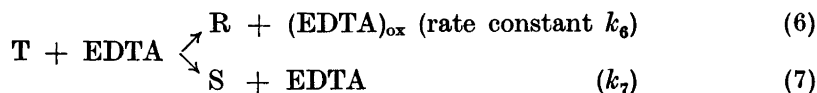
Fig. 3. Formation of semioxidized fluorescein: transient change in optical density observed at 428 mμ after flashing 0.1 μM fluorescein, – – – – in the absence of reactants, and ———— in the presence of 1 mM EDTA and ferric chelate of concentration indicated in the figure. Cell length is 60 cm.

transient changes in optical density at 355 and 428 mμ is due to recombination reactions.

Fluorescein in solutions containing EDTA. The triplet fluorescein formed by flash excitation was found to disappear more rapidly in the presence of EDTA than in its absence, thus indicating that one or both of the following quenching reactions are occurring:

$$T + EDTA \begin{cases} R + (EDTA)_{ox} & \text{(rate constant } k_6) \quad\quad (6) \\ S + EDTA & (k_7) \quad\quad\quad\quad\quad (7) \end{cases}$$

where $(EDTA)_{ox}$ represents the primary oxidation product(s) of EDTA. The rate of decay of the triplet dye immediately after the flash corresponds to a first-order reaction with a rate constant of 1.15×10^3 sec^{-1}, as compared to the value of 1.00×10^3 sec^{-1} found in the absence of EDTA (Fig. 1). The relative error in these measurements is estimated to be less than 5 %, and one obtains thus a value for the quenching constant

$$k_6 + k_7 = (1.5 \pm 0.5) \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$$

The curves in Fig. 2 corresponding to measurements at 355 mμ in the absence and presence of EDTA show that the rate of appearance of semireduced dye and the total amount formed are increased in the presence of the reducing agent, and confirm thus the occurrence of reaction (6). It is calculated from the difference in the heights of the maxima of these curves that reactions (6) and (7) are equally important. However, the accuracy is not very high in these measurements, and reaction (7) may possibly not be occurring at all.
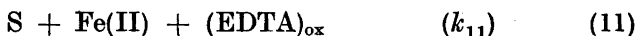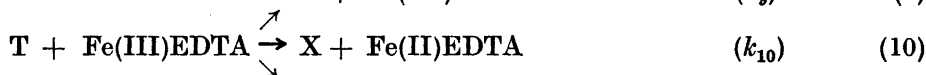
The optical density curve at 428 mμ in the presence of EDTA (Fig. 3) does not directly reveal the appearance of the semioxidized dye. However, if one takes into account the decrease in optical density due to the formation of

the weakly absorbing semireduced dye by reaction (6), one finds that the semioxidized dye is appearing to an appreciable extent (formed by the competing reactions (4) and (5)). This shows that the hypothetical reaction

$$X + EDTA \rightarrow S + (EDTA)_{ox} \tag{8}$$

is not rapid, as will also be confirmed in the following.

Fluorescein in solutions containing EDTA and Fe(III)EDTA. The triplet dye was found to be quenched very efficiently by the ferric chelate. One may postulate three different pathways for the quenching process:

$$S + Fe(III)EDTA \qquad (k_9) \tag{9}$$

$$T + Fe(III)EDTA \rightarrow X + Fe(II)EDTA \qquad (k_{10}) \tag{10}$$

$$S + Fe(II) + (EDTA)_{ox} \qquad (k_{11}) \tag{11}$$

The quenching constant $(k_q = k_9 + k_{10} + k_{11})$ was calculated from the influence of the concentration of ferric chelate on the rate of triplet decay immediately following flashing (Fig. 1). The slope of the line reproduced in Fig. 1 gives

$$k_q = 0.8 \times 10^9 \ M^{-1}sec^{-1}$$

The importance of reaction (10) was estimated from the amount of semioxidized dye appearing after the consumption of the triplet. Fig. 3 shows measurements at 428 mμ at increasing concentrations of ferric chelate. At a ferric concentration of 10 μM the reactions (1)—(5) can be neglected, and the height of the maximum transient change in optical density at 428 mμ then gives the ratio $k_{10}/k_q = 0.15$, that is,

$$k_{10} = 1.0 \times 10^8 \ M^{-1}sec^{-1}$$

When the ferric chelate concentration is increased to 10^{-4} M the production of semioxidized dye is increased due to repeated excitation of dye molecules previously deactivated from the triplet state by the quenching reaction (9) (Fig. 3). The rate of disappearance of the semioxidized dye immediately after the flash is also increased; this rate was found to be roughly proportional to the square of the concentration of semioxidized dye, indicating that a second-order reaction is occurring. Possible second-order reactions consuming the semioxidized dye are

$$2 \ X \rightarrow transient \ product(s) \tag{12}$$
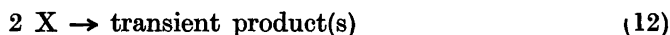
or (since ferrous chelate is formed in equal amounts with the semioxidized dye by reaction (10))

$$X + Fe(II)EDTA \rightarrow S + Fe(III)EDTA \tag{13}$$

In order to determine whether reaction (13) is important, flash runs were performed on solutions containing 10^{-4} M ferrous chelate in addition to the other solutes. Only a minor increase in the rate of disappearance of the semioxidized dye formed during the flash was observed in this case, showing that reaction (13) can be neglected.

The possibility of reaction of the semioxidized dye with EDTA (reaction (8)) was investigated by flash studies of 0.1 μM fluorescein solutions containing 10 μM ferric chelate and EDTA at different concentrations (0.2 $\times$ 10^{-3} — 2 $\times$ 10^{-3} M). The decay rate of the semioxidized dye formed during the flash did not depend upon the concentration of EDTA, confirming that reaction (8) is not occurring.

The appearance of semireduced fluorescein during flashing was also studied as a function of the concentration of ferric chelate, and the results are shown in Fig. 2. At a ferric concentration of 2 μM the semireduced dye is formed only to a very slight extent (by reactions (4)—(6)) and disappears more rapidly than in the absence of the oxidizing agent. This shows that the reaction

$$R + Fe(III)EDTA \rightarrow S + Fe(II)EDTA \qquad (14)$$

is occurring. At a ferric concentration of 10 μM no semireduced dye is appearing. A small permanent decrease in optical density is observed after flashing and is possibly due to the photosensitized reduction of ferric chelate which absorbs slightly at 355 mμ.

Absorption measurements showed that there was no permanent change of the dye after flashing. The reappearance of the strong absorption bands of the dye in the visible was found to proceed at the same rate as the disappearance of the absorption due to the semioxidized dye (at ferric concentrations of 10 μM or higher).

It was observed that the reaction products of the oxidation of EDTA contributed to the rate of disappearance of semioxidized dye, and this made the interpretation of the results complicated. For solutions of 0.1 μM fluorescein, 10^{-4} M Fe(III) and 10^{-3} M EDTA the decay rate of the flash-induced transient absorption at 428 mμ doubled from the first to the second flash, showing that the permanent products of EDTA react with the semioxidized dye. The results presented in the figures are for the first flash.

DISCUSSION

The results show that the photosensitizing effect of fluorescein is closely related to the photoreducing and photooxidizing properties of the dye. The mechanism of photoreduction of dyes by EDTA has been studied extensively[6-12] and it is agreed that generally the triplet dye, formed by intersystem crossing from the excited singlet state, is involved in the reaction. Momose, Uchida, and Koizumi[11] showed in a study of the photoreduction of fluorescein by EDTA in alkaline aqueous solution that the triplet dye is reduced to the semiquinonic radical (abs. max. 394 mμ) which accumulates as a comparatively stable intermediary product. The present study shows that this reaction (reaction (6)) occurs also at pH 5.5, although the semireduced dye in this case is formed in a different ionic form.

It is also known[1] that semireduced dye is formed in an electron transfer reaction between two fluorescein molecules one of which is in the triplet state. The semioxidized dye radical which is simultaneously produced in this reaction may oxidize a reducing agent present (e.g. allyl-thiourea[1]) regenerating

the dye in the ground state. That the latter reaction mechanism is indeed important in the photoreduction of xanthene dyes by allyl-thiourea was confirmed in quantum yield studies reported by Koizumi et al.[11,12] On the other hand it was observed [11] that the dye-dye electron transfer reaction does not contribute to the rate of photoreduction when EDTA is used as reducing agent. This behaviour is explained by the present results, since it was found that the semioxidized dye formed in the dye-dye electron transfer reaction does not react easily with EDTA. The semioxidized dye is instead consumed rapidly by recombination with semireduced dye which is accumulating in the solution as a result of the direct photoreduction of the dye by EDTA. (In the photoreduction by allyl-thiourea the semireduced dye apparently reacts further with the reducing agent and is not accumulating [11] — the recombination is therefore not important in this case.)

It has been shown previously [1,2] that a process analogous to the first step in the photoreduction of fluorescein is occurring in the photo-oxidation of the dye. The triplet state of fluorescein is quenched very efficiently by ferric perchlorate at pH 2 (rate constant 1×10^9 M^{-1} sec^{-1}); the quenching reaction leads to an appreciable extent to the formation of semioxidized dye and Fe(II). The present results show that the chelation of the ferric ion with EDTA does not influence the quenching and oxidizing activities of this ion towards the fluorescein triplet (reactions (9) and (10)). The quenching reaction (11), corresponding to a one-step photosensitized oxidation of EDTA by ferric ion can be excluded, since it has been shown [6,12] that the sensitized photo-oxidation of EDTA is occurring only when EDTA is present in excess of the ferric ion.

It is seen from the preceding that the excited sensitizer may react as well with the oxidizing as the reducing agents present in the photosensitized reaction. However, since the oxidation of the triplet dye by ferric chelate is very efficient compared to the reduction by EDTA ($k_{10}/k_6 \approx 10^3$) the oxidation of the dye is expected to be the first step in the photosensitized reaction (unless EDTA is present in very great excess compared to ferric ion). The semioxidized dye thus formed disappears rapidly in a second-order reaction that apparently does not involve neither EDTA, ferric, or ferrous chelate, and most probably is a dimerization of two semioxidized dye radicals:

$$2 \text{ X} \rightarrow \text{X}_2 \tag{15}$$

Strong kinetic evidence for the possibility of formation of such a transient dimer was presented in a previous study.[1] However, it has not yet been possible to detect an absorption characteristic of the product of reaction (15), and this reaction therefore still must be considered as tentative.

Since the dye was found to be regenerated after flashing at the same rate as the semioxidized dye disappeared one has to conclude that the dimer formed by reaction (15) reacts with EDTA within the time of resolution of the flash apparatus. The overall result of this reaction (which probably proceeds in several steps) may then be expressed as:

$$\text{X}_2 + 2 \text{ EDTA} \rightarrow 2 \text{ S} + 2 \text{ (EDTA)}_{ox} \tag{16}$$
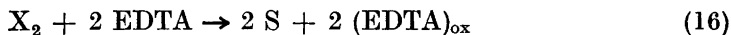
This reaction closes the reaction cycle involved in the photosensitization mechanism. The oxidized EDTA radical reacts further giving stable

products.[8,14] However, the study of these reactions was not the object of the present investigation.

The results thus clearly demonstrate the transient formation of semi-oxidized dye in the photosensitized reaction. Since it is known that other dyes [3,7] may be oxidized reversibly in a one-electron process it is expected that the primary steps of the described sensitization mechanism are of a more general importance.

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