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Elementary processes in the eosin-sensitized photooxidation of 3,3′-diaminobenzidine for correlative fluorescence and electron microscopy

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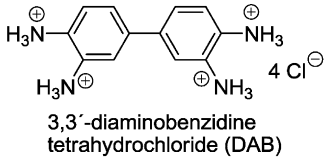
ABSTRACT

The sensitized photopolymerization of 3,3′-diaminobenzidine tetrahydrochloride (DAB) to yield a localized electron-dense precipitate is the basis of a correlative imaging technique, in which fluorescence and transmission electron microscopies are applied to dye-labeled biological samples. In the present work, the eosin (Eo) sensitized photooxidation of DAB has been investigated, as a model system for understanding the complex photochemical mechanism of this imaging process. It was observed that the irradiation with visible light (515 nm) of aqueous solutions of DAB plus Eo triggers a fast photoreaction of DAB, a parallel consumption of dissolved oxygen, and the formation of an optically dense polymer. Time-resolved spectroscopic measurements as a function of solution composition were used to analyze the initial reactive steps of the photoreaction, which are mediated by the Eo lowest excited triplet state (^{3}Eo*). From all these experiments it was concluded that singlet molecular oxygen [$O_2(^1\Delta_g)$], produced by the well-known ^{3}Eo* plus O_2 reaction, and superoxide radical anion ($O_2^{\bullet-}$) are the dominant reactant species in the photoprecipitation reaction. In contrast, in the absence of dissolved oxygen the rate of the photoreaction is only a 15% of the rate determined under aerobic conditions.

1. Introduction

Correlative microscopy is defined as the use of two or more imaging methods to examine the same object [1]. In the case of biological samples, the most common combination involves the use of fluorescence and electron transmission microscopies. In this way, the complementary characteristics of each technique provide much more information than their independent application, as aptly reviewed recently [1]. One of the most successful ways of combining fluorescence and electron microscopies is based on the photooxidation of 3,3′-diaminobenzidine, generally in the form of the more water soluble tetrahydrochloride salt (DAB in the following, see formula) [2–6]. In this method DAB is transformed into a polymer, either by direct or by dye-sensitized irradiation (Scheme 1) [7,8]. The polymer appears as a brown, photostable, electron- and optically dense precipitate, formed by reaction of DAB with reactive oxygen species (ROS). The labeled cell or tissue sample can now be directly imaged by electron microscopy. Alternatively, further chemical reaction of the polymer with osmium tetroxide may be carried out, yielding in situ a reduced osmium black deposit that largely improves the contrast of electron microscopy images. As the lifetimes of the ROS are usually short, DAB is only oxidized in the immediate vicinity of the label, so that this technique ensures that only dye-labeled or dye-stained structures are visualized through the generated electron-dense polymer. In order to avoid secondary reactions of DAB and diffusion of reaction products, the photooxidation must be carried out in the cold, usually at less than 5 °C [6].



3,3´-diaminobenzidine tetrahydrochloride (DAB)

Chemical structure of 3,3´-diaminobenzidine tetrahydrochloride.

Different fluorescent groups have been used as labels for ROS generation [6,9–12], including the green fluorescent protein [13]. The extent of DAB photooxidation can be followed frequently from the changes in fluorescence intensity of the label if the emission decreases by photobleaching, or if it is enhanced by the photoformation of an emissive product [9,14].

The oxidative polymerization of DAB can be accelerated using high intensity light sources, such as those found in confocal laser scanning microscopes, and by working in an atmosphere of pure oxygen [15]. The DAB polymer can also be formed in the dark,

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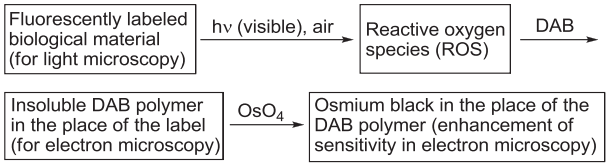

Scheme 1. Main steps in the photoconversion method of correlative imaging by both fluorescence and electron microscopy of dye-labeled biological samples, using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and osmium tetroxide.

by peroxidase-catalyzed oxidation with endogenous or exogenous H_2O_2, as in earliest [7] and more recent [16,17] applications of the method. The chemical structure of the photogenerated polymer is unknown, although the presence of phenazine groups has been proposed from the IR spectra of the material, likely formed through radical reactions via aminoimino quinoid structures [7].

In spite of the increasing use of the DAB photopolymerization reaction, specific applications of the technique are still painfully developed quite empirically. This may be due, in part, to the lack of detailed information on the photochemical reactions that take place in the photooxidation process at the biological sample. To fill this gap, we contribute here with an experimental and mechanistic study of the eosin (Eo)-sensitized DAB photooxidation in water solution in aerobic conditions, as a model of the process that occurs in the biological sample. The dye Eo presents several advantages for correlating light and electron microscopies [3], because this compound is moderately fluorescent, relatively photostable, and an efficient $O_2(^1\Delta_g)$ generator [18].

2. Materials and methods

2.1. Materials

3,3′-Diaminobenzidine tetrahydrochloride (DAB) (dihydrate), deuterium oxide (99.9%, D_2O), the enzyme superoxide dismutase (SOD), eosin Y (Eo), perinaphthenone (PN) and furfuryl alcohol (FFA) were from Sigma–Aldrich Co. and used as received. Water was triply distilled. All the measurements were carried out at room temperature and with freshly prepared solutions.

2.2. Stationary photolysis

Stationary photolysis of aqueous solutions containing DAB and Eo was carried out in a home-made set-up with two commercial green light-emitting diodes as irradiation source, with emission centered at 510 ± 44 nm. The rate constant k_r for the reaction of DAB with $O_2(^1\Delta_g)$ was determined by the method of Scully and Hoigné [19] using a reference compound R and the expression $\text{slope}/\text{slope}_R = k_r[\text{DAB}]/k_{rR}[\text{R}]$, where slope and slope_R denote the slopes of the first-order plot of molecular oxygen consumption by DAB and R, respectively, under PN-sensitized irradiation. Assuming that the reaction of $O_2(^1\Delta_g)$ with the quencher DAB or R is the only way of molecular oxygen consumption, through 1:1 stoichiometry, the ratio of the first order slope of oxygen uptake by DAB and R, each at the same concentration, yields k_r/k_{rR}. The reference compound was furfuryl alcohol (FFA), with a reported k_r value of $1.2 \times 10^8\ M^{-1}\ s^{-1}$ [20]. The rates of DAB photoconsumption in aerobic and argon-saturated solution were obtained from the absorbance decrease at 221 nm, upon Eo-sensitized photolysis, as a function of irradiation time. Oxygen uptake in water solutions was monitored with a 97-08 Orion electrode. Absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer.

2.3. Time resolved $O_2(^1\Delta_g)$ phosphorescence detection (TRPD)

The total quenching rate constant (k_t) for $O_2(^1\Delta_g)$ deactivation by DAB was determined by recording its NIR phosphorescence lifetime. Second harmonic (532 nm) pulses (7 ns, 5 mJ, and 1 Hz repetition frequency) from a Nd:YAG laser (Spectron) were used as the excitation source. The emitted $O_2(^1\Delta_g)$ phosphorescence (1270 nm) was detected using a Judson J16/8Sp Germanium detector, after passing through a 1250-nm interference and two Wratten filters. The output of the detector was coupled to a 400 MHz digital oscilloscope (HP 54504A) and averaged, usually ten times. D_2O was used to increase $O_2(^1\Delta_g)$ lifetime [20].

2.4. Laser flash photolysis experiments

Spectral transients from argon-saturated aqueous solutions of Eo (0.04 mM) were recorded with a home-made flash photolysis apparatus, using the Nd:YAG laser (Spectron) as excitation source (532 nm, 7 ns, 5 mJ, 1 Hz repetition frequency) and a 150-W xenon lamp as analyzing light. The detection system comprised a PTI monochromator and a red-extended photomultiplier (Hamamatsu R666). The signal was acquired and averaged with a digital oscilloscope (Hewlett-Packard 54504).

The decay of the Eo triplet state (^{3}Eo*), generated by the 532-nm laser pulses, was monitored at 570 nm, where the interference from other possible species was negligible. Decay curves were measured at low Eo concentration (0.01 mM), and at low enough laser energy (5 mJ pulse^{-1}) to minimize self-quenching and triplet–triplet annihilation processes. The rate constant for electron transfer from ^{3}Eo* to DAB, k'_{et}, was determined by the Stern–Volmer expression $1/{}^3\tau = (1/{}^3\tau_0) + k'_{et}[\text{DAB}]$, where ${}^3\tau$ and ${}^3\tau_0$ are the experimental lifetimes of ^{3}Eo* in the presence and in the absence of DAB, respectively.

3. Results

3.1. Stationary photolysis

The difference absorption spectrum of a water solution of Eo (91 μM, $A_{516} = 0.54$) plus DAB (0.28 mM) *vs* Eo (91 μM) is shown in Fig. 1, main panel. The observed absorption bands in the 200–250 nm range are largely due to DAB transitions. The visible-light irradiation of the air-equilibrated solution of the former mixture DAB plus Eo gives rise to spectral changes that can be assigned to reaction of DAB and, to a lesser extent, of the dye Eo. After long irradiation times, a suspension of brown particles with structureless absorption spectrum extending from *ca.* 350 nm could be appreciated in the photolysis cell, due to the formation of non-soluble polymeric products from DAB [7,8]. Photoirradiation of the same solution in the absence of dissolved oxygen gives rise to similar spectral changes but with a much lower rate. In fact, the rate of photoreaction under argon-saturated atmosphere only reaches *ca.* 15% of the overall rate determined under aerobic conditions (Fig. 1, inset A). On the other hand, oxygen consumption was not detected when Eo solutions were irradiated in the absence of DAB, whereas noticeable rates of oxygen uptake could be observed upon the addition of DAB (0.20 mM).

3.2. Quenching of ^{1}Eo* and ^{3}Eo*

The fast decay time of the initially excited Eo singlet state (^{1}Eo*) under the experimental conditions used in this work (with a fluorescence lifetime in neutral water solution of 1.3 ns [21]) prevents the direct reaction of this species with DAB in 10^{-3} M solutions.

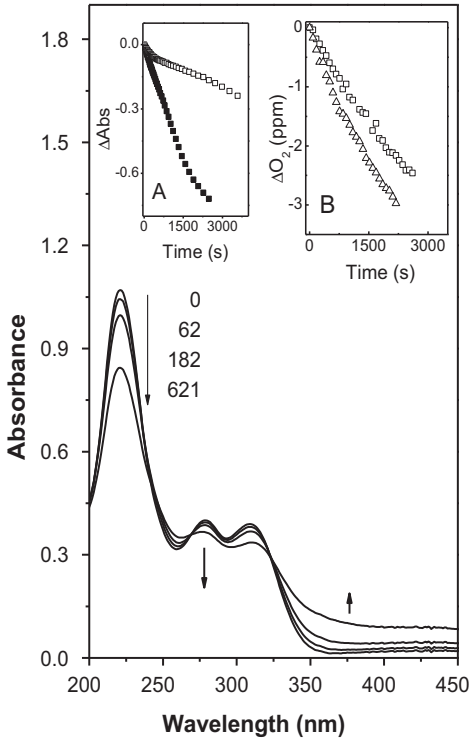

Fig. 1. Main panel: changes in the absorption spectra as a function of the irradiation time (0–621 s) under stationary irradiation (515 nm) of an aqueous solution of eosin (Eo) (91 μM) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.28 mM), after subtraction of the spectrum of Eo (91 μM), under air-equilibrated conditions. Inset A: absorbance changes at 221 nm of the same Eo plus DAB solution as a function of the irradiation time, in aerated (■) or argon-saturated (□) solution. Inset B: changes in dissolved oxygen concentration as a function of the irradiation time of an aqueous solution of Eo (95 μM) and DAB (0.54 mM), with (□) or without (△) the presence of superoxide dismutase enzyme (100 nM).

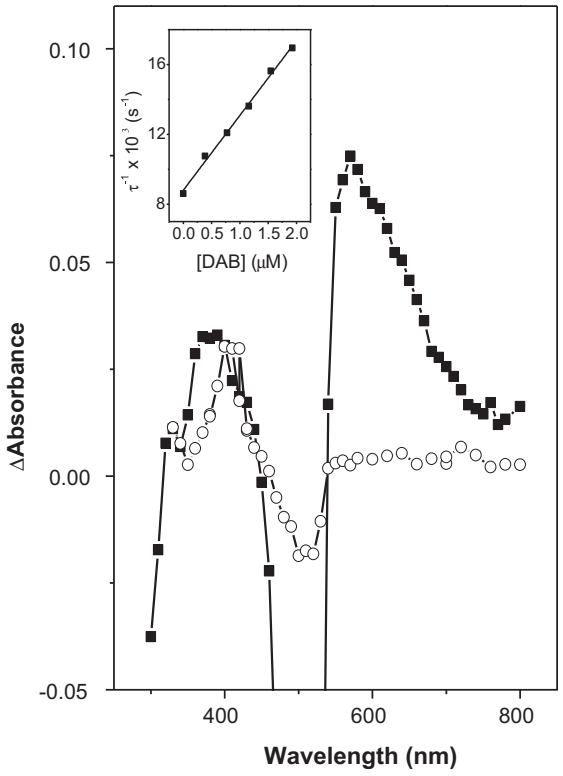

Fig. 2. Transient absorption spectra of an argon-saturated aqueous solution of eosin (Eo) (184 μM) with (○) or without (■) the presence of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.74 mM), registered 2 μs after the excitation laser pulse. Inset: Stern–Volmer plot of the quenching of ^{3}Eo* by DAB in argon-saturated aqueous solution.

The transient absorption spectrum of an Eo solution immediately (2 μs) after the laser pulse (Fig. 2, main panel) is coincident with that of the lowest triplet state of the dye, ^{3}Eo* [22]. On the other hand, the spectrum recorded 2 μs after the exciting laser pulse under the same experimental conditions, but in the presence of DAB (0.74 mM), is similar to that reported for the Eo$^{\bullet-}$ species generated through electron transfer from phenol to ^{3}Eo* in aqueous solution [22]. The triplet lifetime of Eo (116 μs) was neatly reduced in the presence of DAB in the sub-mM concentration range, as a result of strong interaction between ^{3}Eo* and DAB. A value for the bimolecular rate constant k'_{et} of $1.50 \pm 0.03 \times 10^9$ M^{-1} s^{-1} (Scheme 2, process (4)) was graphically derived for this process (Fig. 2, inset).

3.3. The role of $O_2(^1\Delta_g)$

The amino-aromatic structure and known properties of DAB make this compound a likely candidate for $O_2(^1\Delta_g)$ quenching [20]. In D_2O and with Eo as a sensitizer, $O_2(^1\Delta_g)$ phosphorescence was quenched by DAB in the sub-mM concentration range (Fig. 3, inset), with an overall rate constant $k_t = 1.14 \pm 0.05 \times 10^9$ M^{-1} s^{-1}, as derived from a simple Stern–Volmer formalism. This value is an order of magnitude higher than that previously found for the same reaction with DAB–Mn^{++} complex instead of DAB [23]. The $O_2(^1\Delta_g)$–DAB interaction may be a physical process or a reactive process, or a combination of both. For the reactive process, a k_r value of $6.23 \pm 0.08 \times 10^8$ M^{-1} s^{-1} was determined from oxygen uptake measurements in the presence of DAB (Fig. 3, main panel), with FFA as a reference and using PN as a dye-sensitizer. PN is an exclusive $O_2(^1\Delta_g)$ producer, with a reported quantum yield value of *ca.* 1 for the generation of this oxidative species [24]. It was employed in order to avoid possible contributions from oxygen-consuming side reactions due to the potential generation of ROS, other than $O_2(^1\Delta_g)$, by Eo.

3.4. The role of $O_2^{\bullet-}$

The involvement of the $O_2^{\bullet-}$ species in the Eo-sensitized photooxidation of DAB was studied by measuring the oxygen uptake of aqueous solutions of Eo and DAB, as a function of superoxide

$$\text{Eo} + h\nu \longrightarrow {}^1\text{Eo}^* \quad (1)$$

$${}^1\text{Eo}^* \xrightarrow{k_{isc}} {}^3\text{Eo}^* \quad (2)$$

$${}^3\text{Eo}^* + O_2({}^3\Sigma_g^-) \xrightarrow{k_{et}} \text{Eo}^{\bullet+} + O_2^{\bullet-} \quad (3)$$

$${}^3\text{Eo}^* + \text{DAB} \xrightarrow{k'_{et}} \text{Eo}^{\bullet-} + \text{DAB}^{\bullet+} \longrightarrow P_4 \quad (4)$$

$$\text{Eo}^{\bullet-} + O_2({}^3\Sigma_g^-) \xrightarrow{k''_{et}} \text{Eo} + O_2^{\bullet-} \quad (5)$$

$$O_2^{\bullet-} + \text{DAB} \xrightarrow{k_6} P_6 \quad (6)$$

$${}^3\text{Eo}^* + O_2({}^3\Sigma_g^-) \xrightarrow{k_{ET}} \text{Eo} + O_2({}^1\Delta_g) \quad (7)$$

$$O_2({}^1\Delta_g) \xrightarrow{k_d} O_2({}^3\Sigma_g^-) \quad (8)$$

$$O_2({}^1\Delta_g) + \text{DAB} \xrightarrow{k_q} O_2({}^3\Sigma_g^-) + \text{DAB} \quad (9)$$

$$O_2({}^1\Delta_g) + \text{DAB} \xrightarrow{k_r} P_9 \quad (10)$$

Scheme 2. Elementary steps in the eosin (Eo) photosensitized oxidation of 3,3′-diaminobenzidine tetrahydrochloride (DAB). P denotes a reaction product.

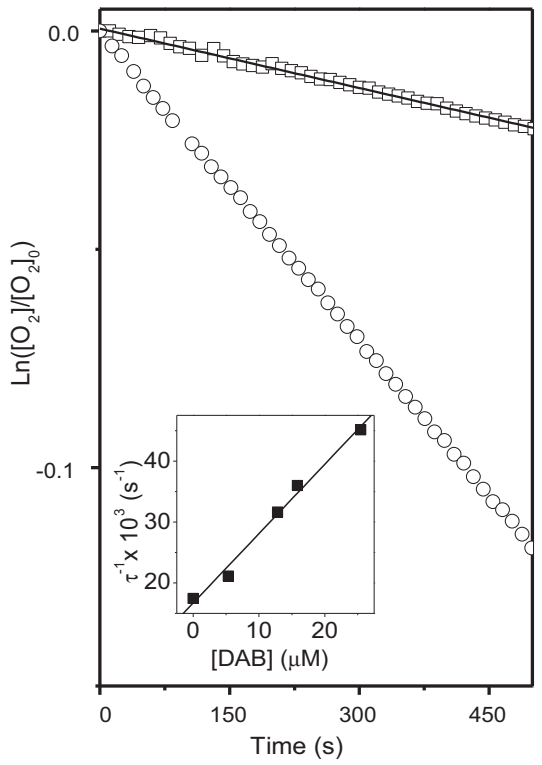

Fig. 3. First order plot of oxygen uptake as a function of time upon visible light irradiation (515 nm) of aqueous solutions containing perinaphthenone (57 μM) and either furfuryl alcohol (0.46 mM) (□) or DAB (0.46 mM) (○). $[O_2]_0$ and $[O_2]$ denote the initial and the actual oxygen concentration, respectively. Inset: Stern–Volmer plot of the quenching of $O_2(^1\Delta_g)$ phosphorescence as a function of DAB concentration (0–27 μM range) in air equilibrated D_2O solution.

dismutase (SDO) concentration. SOD is a well-documented specific $O_2^{\bullet-}$ scavenger, used to confirm/discard the participation of the superoxide anion radical in oxidative events [25,26], based on the reaction

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} O_2(^3\Sigma_g^-) + H_2O_2$$

We have communicated before [27] that the lifetime of $O_2(^1\Delta_g)$ in D_2O solution is not affected by the presence of the enzyme SOD in the 100 nM concentration level. The irradiation of an aqueous solution of Eo (0.095 mM) and DAB (0.2 mM) in the presence of SOD (100 nM) gives rise to a clear decrease of oxygen consumption, with regard to the same experiment in the absence of SOD (Fig. 1, inset B), pointing to the effective participation of the $O_2^{\bullet-}$ species in the photooxidation reaction.

4. Discussion

The kinetic and mechanistic aspects of the Eo-sensitized photooxidation of DAB can be analyzed by reference to a minimum set of elementary reaction steps listed in Scheme 2. In brief, the initially generated Eo singlet excited state (^{1}Eo*, process (1)) converts to ^{3}Eo* by efficient intersystem crossing (process (2)). ^{3}Eo* can be quenched by ground state oxygen, $O_2(^3\Sigma_g^-)$, generating $O_2^{\bullet-}$ by electron transfer (process (3)), or react with DAB molecules to yield the $DAB^{\bullet+}$ radical (process (4)). The radicals formed in these reactions can regenerate the starting compounds by back electron transfer or give rise to products P_4 by irreversible processes.

Regarding the electron transfer process (4), it should be pointed out that while DAB is a good electron donor (0.525 V (NHE)) [28], ground state Eo is relatively difficult to oxidize and to reduce, with described values in water of $(Eo/Eo^+) = 1.1$ V and $Eo(Eo/Eo^-) = 0.8$ V (NHE) [29]. However the reductive quenching of ^{3}Eo* (1.98 eV) by DAB is exergonic, with a driving force for process (4) of 1.76 eV [29].

The semireduced Eo species, $Eo^{\bullet-}$, can react with molecular oxygen [30] yielding $O_2^{\bullet-}$ (process (5)), that can subsequently react with DAB leading to products P_6 (process (6)). ^{3}Eo* can be quenched by oxygen, generating $O_2(^1\Delta_g)$ through the energy transfer process (7). $O_2(^1\Delta_g)$ can be physically scavenged either by solution components (process (8)) or by DAB (process (9)), or can react either with Eo (not shown) or with DAB (process (10)). The rate constant k_t accounts for the overall quenching processes of $O_2(^1\Delta_g)$ by DAB, $k_t = k_q + k_r$.

The involvement of $O_2(^1\Delta_g)$ in the photooxidation of DAB has been demonstrated before using merocyanine 540 as a sensitizer [31], a dye that generates $O_2(^1\Delta_g)$ with very low quantum yield, $<10^{-2}$ [32]. On the other hand, it has been also established [20] that DAB reacts with $O_2^{\bullet-}$, generated in situ with potassium superoxide, to yield an oxidized DAB polymer through a reaction which is favored by Mn^{++} ions, and that this process may compete with the spontaneous dismutation of $O_2^{\bullet-}$ to $O_2(^1\Delta_g)$ and H_2O_2 (rate constant 1.0×10^5 M^{-1} s^{-1}). The NIR phosphorescence of $O_2(^1\Delta_g)$ is quenched by the DAB–Mn^{++} complex with a k_t value of 1.7×10^8 M^{-1} s^{-1}, and by $O_2^{\bullet-}$, with a rate constant close to the diffusion limit, 1.6×10^9 M^{-1} s^{-1} [23].

In the case of the Eo-sensitized photooxidation of DAB studied here, the experiments presented above indicate that the aromatic tetraamine participates in reactive processes with electronically excited states of the dye, as well as with reactive oxygen species. Taking into account the rate constant value of the electron transfer process from DAB to ^{3}Eo* ($k'_{et} = (1.50 \pm 0.03) \times 10^9$ M^{-1} s^{-1}, process (4), i.e. close to the diffusion limit), and the lack of ^{1}Eo*–DAB interactions, the main photodegradation pathways of DAB should involve ^{3}Eo* and the reactive oxygen species produced from this triplet state. Xanthene dyes such as Eo and Rose Bengal are well-known photosensitizers for $O_2(^1\Delta_g)$ generation [18,24,33] and have been used as radical photoinitiators for polymerization reactions in the presence of amines [34]. The photochemical properties of these dyes and the transient species involved under visible light irradiation have been extensively studied [35].

In the stationary photolysis experiments in aerated water solution reported here, with a DAB concentration close to 0.4 mM and about the same value for the dissolved oxygen concentration [36], the generation rates of $O_2(^1\Delta_g)$ (process (7)) and $Eo^{\bullet-}$ (process (4)) depend on the respective rate constant values. For process (7), a k_{et} value of 7×10^8 M^{-1} s^{-1} [37], equivalent to 1/9 of the singlet-oxygen diffusion-controlled rate constant value in water, may be assumed, while the experimentally determined rate constant value for process (4), k'_{et}, is 1.50×10^9 M^{-1} s^{-1}. Hence, for the case of similar generation efficiency of $O_2(^1\Delta_g)$ (process (7)) and $O_2^{\bullet-}$ (process (5)), the relative concentration of $O_2^{\bullet-}$ under photostationary irradiation conditions should be *ca.* twice the concentration of $O_2(^1\Delta_g)$. On the other hand, the interaction of ^{3}Eo* with oxygen would lead largely to $O_2(^1\Delta_g)$-generation (process (7)), due to the comparatively low rate constant for direct $O_2^{\bullet-}$ generation from ^{3}Eo* (process (3), with a reported k_{et} value lower than 10^7 M^{-1} s^{-1} [38]). Nevertheless, the observed decrease of oxygen uptake rate and, consequently, of photoproduct generation, upon Eo-sensitized irradiation of DAB in the presence of SOD (Fig. 1, inset B), confirms the involvement of the species $O_2^{\bullet-}$.

It is known that $O_2^{\bullet-}$ reacts with aromatic amines through a deprotonation–oxidation mechanism rendering azobenzenes (mainly) and nitroso compounds [39]. The related compound *o*-phenylendiamine is effectively oxidized by thermally generated $O_2^{\bullet-}$ in benzene or toluene [40]. In the absence of oxygen, ^{3}Eo* likely reacts with DAB (Fig. 1, inset A) to yield initially $DAB^{\bullet+}$, generated in the electron transfer process (4), and finally radical polymerization products.

The $O_2(^1\Delta_g)$-mediated photooxidation quantum efficiency Φ_r can be estimated from the expression $\Phi_r = k_r[DAB]/(k_d + k_t[DAB])$, in which k_d is the singlet oxygen decay rate constant (process (8)). Nevertheless, the determination of Φ_r includes the knowledge of the actual concentration of the photooxidizable substrate, represented by DAB in this case. A simpler and useful approach is the evaluation of the k_r/k_t ratio, which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation. The calculated value of k_r/k_t is 0.55, indicating an important contribution of $O_2(^1\Delta_g)$ to the effective DAB photooxidation. Aromatic amines frequently behave as physical and chemical scavengers of $O_2(^1\Delta_g)$ [20]. In the particular case of *o*-phenylendiamine, k_t values of 3.4×10^9 and $1.1 \times 10^9\ M^{-1}\ s^{-1}$ have been reported in solution in EtOH [41] and EtOH–H_2O, 1:1 [42], respectively, the latter value in coincidence with the k_t value herein found for DAB in H_2O solution.

5. Conclusions

The visible light-irradiation of an air-equilibrated aqueous solution of Eo and DAB gives rise to a large consumption of oxygen and the efficient transformation of DAB into a non-soluble optically dense polymeric product. The rate of this process is significantly inhibited in the presence of superoxide dismutase, a scavenger of the species $O_2^{\bullet -}$. It is shown here that the photooxidation of DAB is carried out by the strongly oxidant species $O_2^{\bullet -}$ and $O_2(^1\Delta_g)$, both generated through the interaction of $^3Eo^*$ with DAB and oxygen molecules. Kinetic evidence suggests a 2:1 ratio for the respective concentrations of $O_2^{\bullet -}$ and $O_2(^1\Delta_g)$, under photostationary irradiation conditions and similar concentrations of DAB and dissolved oxygen. Direct reaction between $^3Eo^*$ and DAB to yield the photopolymer is a low efficiency process. Similar photooxidation reactions are likely to occur in the imaging technique based on DAB photopolymerization with Eo-labeled biomolecules.

Acknowledgments

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