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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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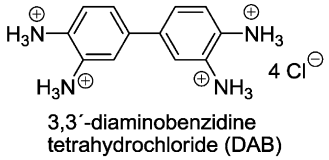
ABSTRACT

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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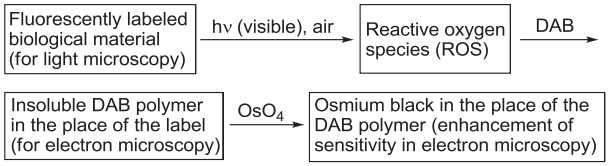

Scheme 1. Main steps in the photoconversion method of correlative imaging by both fluorescence and electron microscopy of dye-labeled biological samples, using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and osmium tetroxide.

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.