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Fringelite D, a Model of the Protist Photosensory Pigments of the Stentorin and Blepharismin Types: The Hypericin and Fringelite D Photosensitized Destruction of Bilirubin

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Summary. Using the hypericin and fringelite D photosensitized destruction of bilirubin together with fluorescence spectroscopy it was found that in contrast to fringelite D hypericin behaves as an effective photodynamic agent producing mainly singlet oxygen. This makes fringelite D and concomitantly the related stentorin and blepharismin pigments better suited for the photosensory transduction chain where, as shown recently, an initial proton expulsion reaction plays the fundamental role. Thus, in organisms using these photosensory pigments the production of deleterious oxygen species becomes diminished as compared to hypericin. In addition it was found that complexation with albumin further inhibits bilirubin destruction.

Keywords. Singlet oxygen; Superoxide radical; Phenanthroperylene quinones; Sensitization; Evolution.

Introduction

Among other reactive oxygen species, singlet oxygen and the superoxide ion are known to be almost ubiquitously produced in living cells. Besides their deleterious effects they can act also as messenger molecules in a variety of cellular processes [1]. Since it is well known that pigments containing the phenanthroperylene quinone chromophore effectively photosensitize the formation of such oxygen species, and in particular the formation of singlet oxygen [2], they might in principle play some role in the photosensory transduction chain of protists like *Stentor coeruleus* or *Blepharisma japonica*. These organisms use the above chromophor within stentorin (**1**) or blepharismin (**2**) as their photosensory pigments [3, 4]. In their native forms they are associated with certain proteins.

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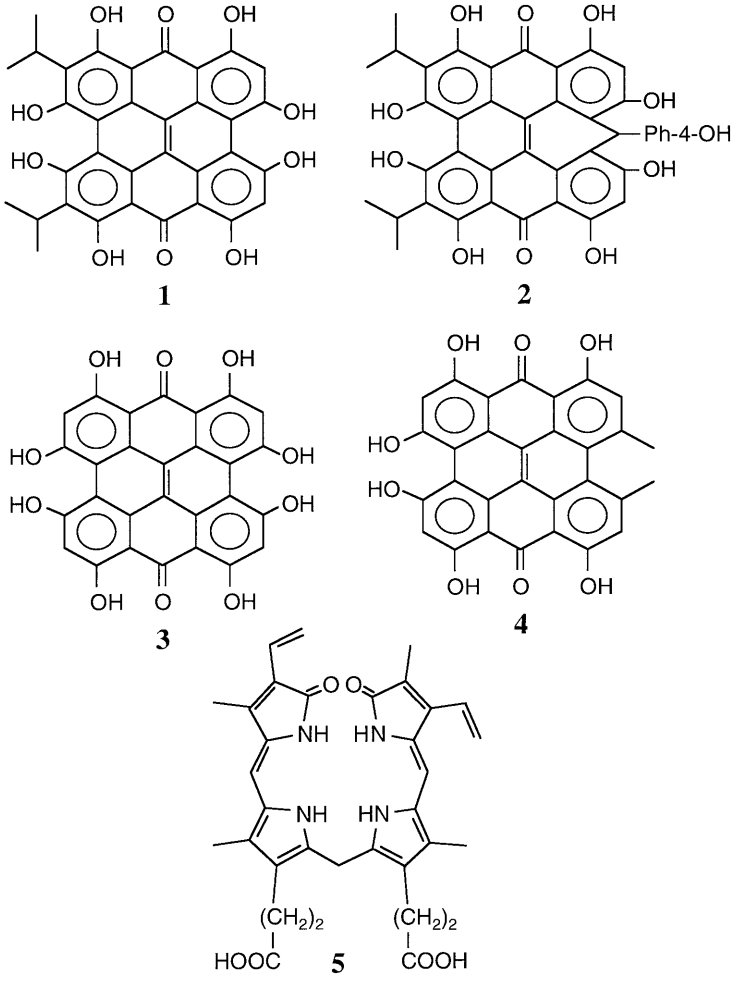

Formulae 1

For the primary event of phototransduction the photoinduced proton expulsion has been corroborated as the favorite candidate in a recent investigation of fringelite D (**3**) by means of two-photon epifluorescence microscopy [5]. In addition, it has been more or less excluded by the results of a videomicroscopic study of the effect of the singlet oxygen quencher crocetin on the photobehaviour of *Blepharisma* that, although blepharismin-sensitized photodynamic reactions, occur, they do not play a meaningful role in initiating photosensory processes [6]. However, the high quantum yield of singlet oxygen sensitization by hypericin (**4**; $\Phi_\Delta \approx 0.4$ [7]), which has been used hitherto as a model of the photosensory pigments, prompted us to investigate the photodynamic behavior of **3**. With four *bay*-hydroxyl groups instead of two it is a system structurally more similar to the natural pigments **1** and **2** than is **4**. For the latter, the photodestruction of bilirubin (**5**) has been given recently an easily accessible quantitative estimate for the production of singlet oxygen [8]. Accordingly, the photodynamic activity of **3** and **4** with respect to **5** will be compared in this account.

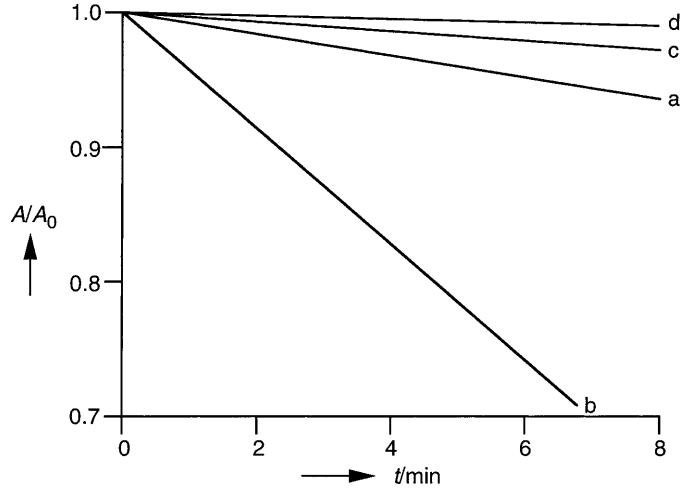

Fig. 1. Normalized absorption (A/A_0) at $\lambda = 453$ nm *vs.* time of a solution of disodium fringelite D ($^{(3-,10-)}$**3**)+disodium bilirubinate ($\mathbf{5}^{2-}$) (a), sodium hypericinate ($^{(3-)}$**4**)+$\mathbf{5}^{2-}$ (**b**), and $^{(3-,10-)}$**3**+$\mathbf{5}^{2-}$+NaN_3 (c) in 80% ethanol, and the human serum albumin complex of $^{(3-,10-)}$**3**+$\mathbf{5}^{2-}$ in aerated phosphate buffer ($pH = 6.9$) (d) upon irradiation at $\lambda > 570$ nm

Results and Discussion

When fringelite D is dissolved in aqueous ethanol at physiological *pH* values, both *bay*-regions become deprotonated as is obvious from the absorption spectrum of such solutions and the two dissociation pK_a values of 1.4 and 3.3 in 20% *DMSO* [9]. As illustrated in Fig. 1, irradiation of a solution of the disodium salt of fringelite D ($^{(3-,10-)}$**3**) together with the disodium salt of bilirubin ($\mathbf{5}^{2-}$) at wavelengths where only the phenanthroperylene quinone absorbs (>570 nm) led to an only slight photodestruction of $\mathbf{5}^{2-}$ (trace a). The rate of this photodestruction was found to be dramatically reduced as compared to the photodestruction of the bilirubinate ion $\mathbf{5}^{2-}$ in case hypericinate ($\mathbf{4}^{-}$) is used as the sensitizer pigment (trace b of Fig. 1). In this case, only one available *bay*-region is known to be deprotonated under the experimental conditions [2]. Thus, a fundamental difference not yet considered in the photochemical behavior of the pigments in which only one ionized *bay*-region is available and such where both *bay*-regions are ionized under physiological *pH* was revealed in this experiment.

In principle, the reason for this difference might be found in differences between the excited states of the dideprotonated pigment **3** and the monodeprotonated species **4**. Therefore, the fluorescence spectra of $^{(3-,10-)}$**3** and $^{(3-)}$**4** were recorded using equimolar solutions and excitation at 584 or 550 nm where the absorptions of the two solutions were found to be equal. Integration of the fluorescence curves thus obtained and referencing to the known fluorescence quantum yield of $^{(3-)}$**4** of 0.23 [10] resulted in a quantum yield of 0.17 for $^{(3-,10-)}$**3**, which is slightly smaller than that of $^{(3-)}$**4**, but hardly significantly defferent enough to account for the observed difference in the photosensitized bilirubin destruction. Hence, according to *Ermolev*'s rule ($\Phi_f + \Phi_{S\rightarrow T} = 1$ [11]), the intersystem crossing rate populating the oxygen sensitizing triplet will be the same or very similar for both compound types.

To check whether in both systems mainly singlet oxygen is the species destroying bilirubinate $\mathbf{5}^{2-}$, the singlet quencher sodium azide [12] was added. As illustrated in Fig. 1, trace c, this species very effectively quenched the photodestruction of $\mathbf{5}^{2-}$ as has been also observed recently in the case of the hypericinate ion $^{(3-)}\mathbf{4}$ [8]. According to this experiment the species leading mainly to the fringelite D induced minor photodestruction of bilirubinate was also found to be singlet oxygen.

From these experiments it might be concluded that, although the triplet states were formed to a similar extent in $^{(3-,10-)}\mathbf{3}$ and $^{(3-)}\mathbf{4}$, the double charge and thus a fundamentally different molecular electrostatic potential in the former as compared with the latter might have prevented the former from an effective interaction with the oxygen molecule to produce singlet oxygen by means of an excitation transfer.

The natural pigments **1** and **2** are associated to certain proteins in their native states [2]. To model this state to some extent, deprotonated fringelite D ($^{(3-,10-)}\mathbf{3}$) was complexed with human serum albumine, which was known from earlier studies to result in a similar complex structure as derived for the hypericinate ion ($^{(3-)}\mathbf{4}$) [9, 13]. Addition of bilirubinate $\mathbf{5}^{2-}$ to this complex then led to the mixed complex of human serum albumin with fringelite D and bilirubin ions ($^{(3-,10-)}\mathbf{3}+\mathbf{5}^{2-}$) as described recently for hypericin (**4**) [8]. As has been demonstrated in the case of the hypericinate ion [8], the photodestruction of fringelitediate coassociated bilirubinate ($\mathbf{5}^{2-}$) was also more or less completely inhibited as shown in Fig. 1, trace d.

Conclusions

Whereas hypericin (**4**) is an efficient sensitizer of singlet oxygen leading to the celebrated photodynamic action of this pigment [2], this quality is dramatically reduced in fringelite D (**3**) which may serve as a model the natural pigments stentorin (**1**) and blepharismin (**2**). Although the two natural pigment series **1** and **2** *vs*. **4** look rather similar at a first glance, they behave in a fundamentally different way due to their different *bay*-regions. In **1** and **2**, both *bay*-regions are deprotonated under physiological conditions, whereas in **4** only deprotonation at the hydroxylic *bay*-site will be possible. This should lead to a different electric potential molecular surface of the resulting excited triplet species, which obviously prevents its interaction with dissolved oxygen. Accordingly, nature evolved two structurally rather similar systems, differing structurally only in minor details but behaving very different with respect to their interaction with light and oxygen. One of these systems (**4**) became a potent photodynamic agent protecting certain plants. The other system (**1**, **2**), which displays a highly reduced photodynamic activity, became the photosensory pigments of certain protists, mainly using light induced proton expulsion for the primary step of photosensory signal transduction.

Experimental

Fringelite D (**3**) [9] and hypericin (**4**) [14] were prepared as described. The purification of **3** and **4** was achieved according to the procedure reported in Ref. [15]. Bilirubin (**5**) was of commercial origin (Sigma) and was purified according to the procedure of *McDonagh* [16]. The pigments **3**, **4**, and **5** were dissolved in 80% aqueous ethanol (p.a., Merck) and brought to *pH* 8 by means of ethanolic

NaOH. Aerated solutions were prepared by bubbling with air (purified over active charcoal) for 5 min prior to use. Sodium azide was of p.a. Merck quality. The human serum albumin complex of **3** was prepared *in situ* by first adding an equimolar amount of the disodium salt of **3** dissolved in a minimum amount of 0.1 *M* KOH to a solution of human serum albumin (97–99%, 1×crystallized and lyophylized; Sigma) in phosphate buffer ($pH = 7$); cf. Refs. [9, 12]. Addition of the disodium salt of **5** resulted in the corresponding mixed human serum albumin/$\mathbf{3}^{2-}$/$\mathbf{5}^{2-}$ complex. Irradiation of the thermostatted samples ($c_{\text{pigment}} = 2 \cdot 10^{-6}\,\text{mol} \cdot \text{dm}^{-3}$, $c_{\text{bilirubin}} = 9 \cdot 10^{-6}\,\text{mol} \cdot \text{dm}^{-3}$, $25 \pm 1°\text{C}$) contained in SiO_2 cuvettes ($d = 1\,\text{cm}$) was performed using a 300 W tungsten lamp and a cut-off filter effectively blocking light below 570 nm. After each irradiation the samples were bubbled with air again. UV/Vis spectra were recorded with a Hewlett Packard 8453 photodiode array spectrometer. Fluorescence spectra were recorded with a Hitachi F-4010 spectrofluorimeter using 80% ethanol of *für die Fluoreszenzspektroskopie* grade (Merck) as the solvent.

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