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# On the Photochemical Proton Expulsion Capability of Fringelite D – A Model of the Protist Photosensory Pigments of the Stentorin and Blepharismin Types

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**Summary.** Using simultaneous two-photon excitation of fringelite D and a fluorescence indicator embedded in a vesicle system it was demonstrated that after excitation a proton was transferred from the pigment to the indicator similarly as recently documented for hypericin. Semiempirical AM1 calculations were used to show that the radical species formed by electron transfer from the excited pigment state constitutes an acid which is therefore well suited for intermolecular proton transfer. Accordingly, this process constitutes a suited candidate for the primary photoprocess in the signal transduction cascade of the photosensory pigments of the stentorin and blepharismin type.

**Keywords.** Two-photon excitation; Intermolecular proton transfer; Epifluorescence microscopy; Phenanthroperylene quinones.

## Introduction

Stentorin (**1**) is a representative of the phenanthroperylene quinone series and represents the phototaxis photoreceptor pigment of the flagellated protozoan *Stentor coeruleus*. A related photoreceptor pigment system comprises the blepharismins and oxyblepharismins of *Blepharisma* [1]. Although there is a wealth of investigations on these systems available, the molecular basis and the primary events following excitation of the pigments are still open questions. In principle, a photoinduced proton or electron transfer could be regarded as the primary process of the signal chain [2].

In the related phenanthroperylene pigment hypericin (**2**), which constitutes a highly active photodynamic agent leading to its potential use in photodynamic therapy [1], it has been shown that two primary processes might be responsible for its physiological action: photosensitized formation of singlet oxygen and superoxide ion on the one hand [3, 4] and proton expulsion following excitation on the other hand [5–7]. Epifluorescence microscopy detected expulsion of a proton following excitation proved to be a valuable and convenient means to assess this latter

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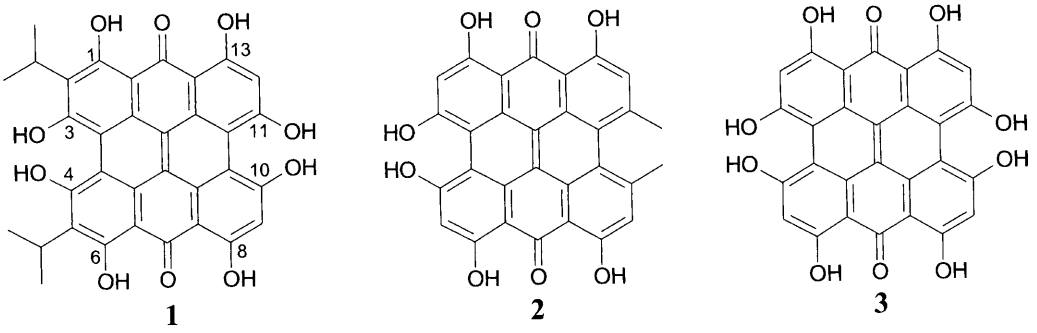


phenomenon [6]. Therefore we now set out to explore this route for photoreceptor pigments choosing fringelite D (**3**) as the fundamental model. This choice stemmed from the fact that **3** is devoid of the alkyl substituents at the aromatic rings, which are thought to play only a minor role for the photochemical behavior of these pigments. However, it comprises, in addition to the four *peri*-hydroxyl groups, the two pairs of *bay*-hydroxyl groups which are a common feature of the *Stentor* and *Blepharisma* photosensory pigments.

## Results and Discussion

In the experiments the phenanthroperylene quinone pigments **2** and **3** were embedded into dipalmitoylphosphatidylcholine vesicles. An aqueous buffer solution of the indicator 2′,7′-*bis*-(2-carboxyethyl)-5(6)-carboxy-fluorescein (**4**) was encapsulated into them and surrounded them as well. The *pH* value was adjusted to 7.1 because it was estimated from a spectrofluorimetric titration that this was the region of highest indicator sensitivity. In addition, it has to be noted that the pigments **2** and **3** were deprotonated under these conditions due to the high acidities of the phenolic groups in their *bay*-regions [8–10]. According to their $pK_a$ values of 0.8 and 2.9 (**1** [8]), 1.8 (**2** [9]), and 1.4 and 3.3 (**3** [10]), the species were present as $\mathbf{2}^-$ and $\mathbf{3}^{2-}$ under the given conditions. Using the experimental setup displayed in Fig. 1 the

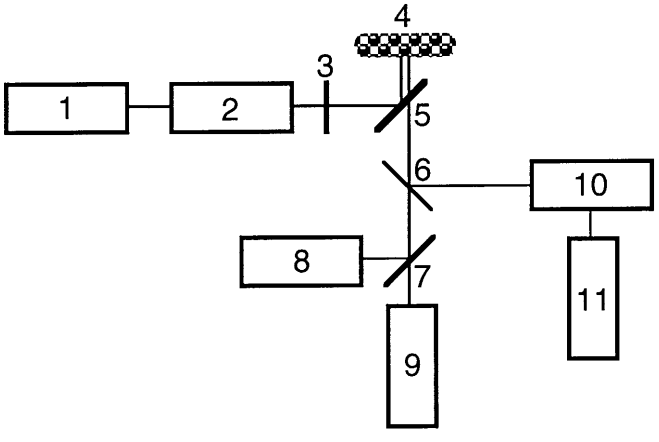


**Fig. 1.** Schematic experimental setup: 1. . .femtosecond laser, 2. . .acousto optic modulator, 3. . .pinhole, 4. . .sample on slide of the epifluorescence microscope, 5. . .dichroic filter, 6. . .flipable mirror, 7. . .dichroic, 8. . . and 9. . .avalanche photodiodes, 10. . .monochromator, 11. . .photomultiplier tube

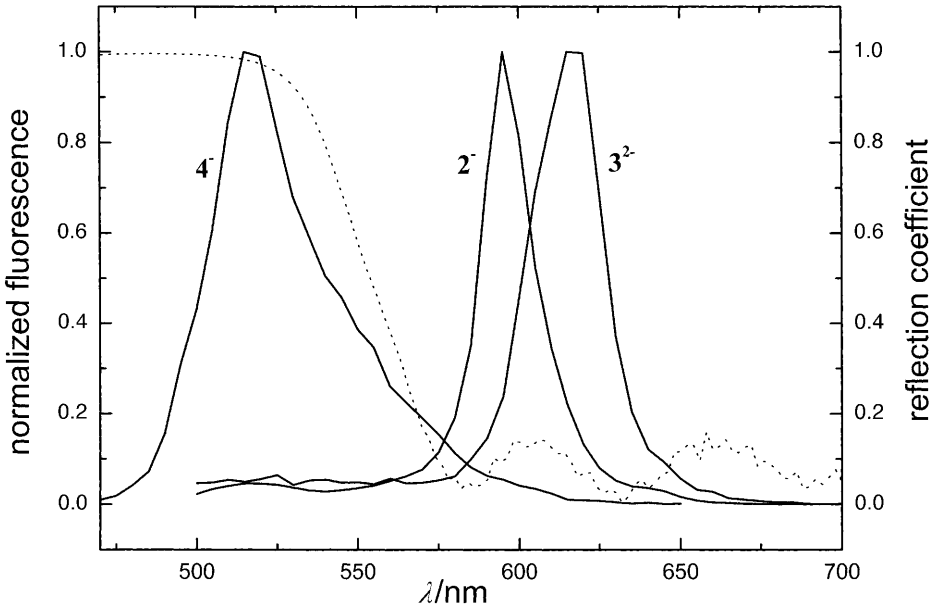


**Fig. 2.** Two-photon excited fluorescence spectra (left scale) of $\mathbf{2}^-$, $\mathbf{3}^{2-}$, and $\mathbf{4}^-$ dissolved in 80% aqueous ethanol at *pH* 7 (—), and reflection coefficient (right scale) of the dichroic mirror (···; cf. Fig. 1, #7)

phenanthroperylene quinone derivatives and the indicator $\mathbf{4}^-$ were simultaneously excited by means of a non-linear two-photon event [11] with laser light of 850 nm, and the resulting fluorescence of $\mathbf{4}^-$ ($F$) at 500–550 nm was recorded in dependence of the mean irradiation power within 10 ms.

The two-photon excitation is superior to one-photon excitation used earlier [6] due to its smaller excitation volume requirements, the necessity of using one laser only, and, in peculiar, to its complete absence of background radiation in the fluorescence observation wavelength range. In addition, the fluorescence of the pigment could be observed simultaneously with the fluorescence of the indicator using a dichroic mirror (7 in Fig. 1). To test its utility for the present purpose, its reflection coefficient was measured. It is displayed in Fig. 2 together with the fluorescence spectra of $\mathbf{2}^-$, $\mathbf{3}^{2-}$, and $\mathbf{4}^-$ resulting from two-photon excitation at 850 nm. Another superiority of this kind of experiment lies in the fact that artifacts are negligible due to the simultaneous excitation of pigment and indicator. Using this arrangement, the fluorescence of the pigments $\mathbf{2}^-$ and $\mathbf{3}^{2-}$ were effectively separated from the fluorescence of the indicator anion $\mathbf{4}^-$ and were focused onto the detector channels 9 and 8 (Fig. 1). Accordingly, the two emissions could be observed independently from each other. Vesicles containing only the pigment $\mathbf{2}^-$ or $\mathbf{3}^{2-}$ were also measured as controls and no significant difference to the fluorescence behavior of **2** and **3** in the mixed vesicles could be detected. Alternatively, measuring vesicles containing only the indicator $\mathbf{4}^-$ yielded its intrinsic fluorescence $F_0$ under the experimental conditions.

Figures 3 and 4 display the results obtained on $\mathbf{2}^-$ and $\mathbf{3}^{2-}$. As could be derived from them, the logarithm of the fluorescence intensity of the indicator $\mathbf{4}^-$ (1.86) as

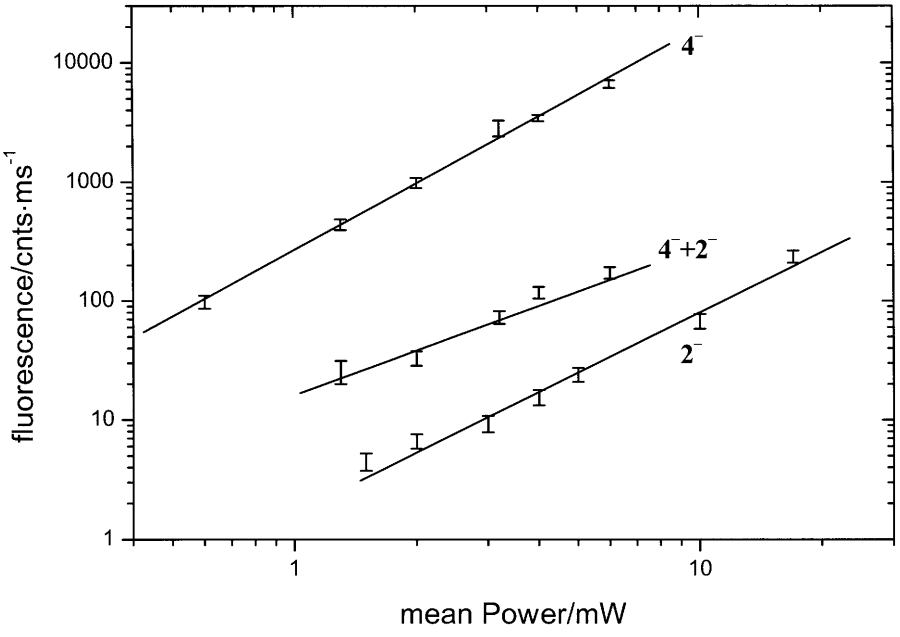


**Fig. 3.** Irradiation power dependence of the fluorescence intensity of ionized hypericin ($\mathbf{2}^-$), indicator ($\mathbf{4}^-$), and the fluorescence of $\mathbf{4}^-$ in $\mathbf{2}^- + \mathbf{4}^-$ in dipalmitoylphosphatidylcholine vesicles

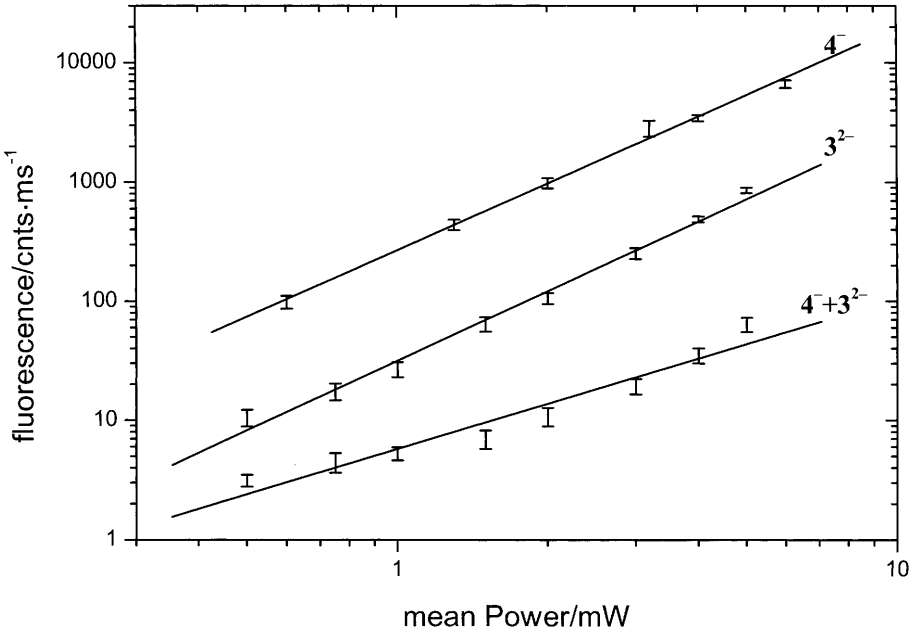


**Fig. 4.** Irradiation power dependence of the fluorescence intensity of ionized fringelite D ($\mathbf{3}^{2-}$), indicator ($\mathbf{4}^-$), and the fluorescence of $\mathbf{4}^-$ in $\mathbf{3}^{2-} + \mathbf{4}^-$ in dipalmitoylphosphatidylcholine vesicles

well as those of the pigments $\mathbf{2}^-$ (1.68) and $\mathbf{3}^{2-}$ (1.94) displayed a slope of about 2 in their dependence on the power logarithm, a behavior which is characteristic of two-photon processes (the fluorescence intensity $F$ and the illumination intensity $I$ in two-photon excitation are related by $F = I^2$, thus $\log F = 2\log I$ [12]). The somewhat smaller slope obtained in the case of $\mathbf{2}^-$ might be related to association effects within the vesicles. In the vesicles containing indicator as well as the pigment, this slope became significantly smaller (1.24 and 1.26 in case of $\mathbf{2}^-$ and $\mathbf{3}^{2-}$), indicating the transfer of protons from a species generated by pigment excitation to the indicator anion $\mathbf{4}^-$. The protonation of the indicator anion diminishes its fluorescence intensity in comparison to the control experiment without $\mathbf{2}^-$ or $\mathbf{3}^-$. In addition, the absolute value of the indicator fluorescence $F$ was considerably diminished as compared to the intrinsic indicator fluorescence intensity $F_0$ measured for the vesicles without the pigment. Thus, protonation of the indicator became evident from two effects: the released protons diminished the fluorescence of the indicator, and this diminution was itself power dependent as indicated by the smaller slope of the signal–power dependence. Accordingly, both effects observed were similar for $\mathbf{2}^-$ and $\mathbf{3}^{2-}$, thus pointing to a similar or even the same mechanism of intermolecular proton transfer from the pigments $\mathbf{2}^-$ and $\mathbf{3}^{2-}$ to the indicator $\mathbf{4}^-$ following their excitation.

With respect to the hypericinate ion ($\mathbf{2}^-$) it has been deduced recently that an electron is transferred from the excited singlet or the triplet state to another hypericinate ion creating a hypericin radical $\mathbf{2}^{\cdot}$. This radical has been shown not to be of a radical-zwitterion type by means of AM1 calculations as suggested before [6], but to be a true radical species [7]. It has been also found to be of comparable acidity to hypericin itself and thus is apt to transfer a proton to the indicator [7].

Because the two-photon excitation results were similar for the hypericinate ion ($\mathbf{2}^-$) and the di-deprotonated fringelite D ($\mathbf{3}^{2-}$), AM1 calculations [13] of the various species of **3** and **1** were undertaken to clarify the mechanistic details and fundamentals (in the following the position from which a proton was abstracted will be indicated by a superscript in parentheses on the left side of the formula number and its state (charges: $^+$, $^-$; radical: $^{\cdot}$) as superscript on its right side).

Starting with fringelite (**3**), the most stable tautomer was found to be the 7,14-dixo system, and its conformation was derived to be the propeller one ($\Delta H_f = -1221\,\mathrm{kJ \cdot mol^{-1}}$). This was in agreement with the results obtained for hypericin (**2**) [7] and earlier force field calculations on **3** [14]. The same was the case with all ionized and radical species discussed below. Deprotonation was found to be most favorable in its *bay* position leading to $^{(3)}\mathbf{3}^-$ ($\Delta H_f = -1464\,\mathrm{kJ \cdot mol^{-1}}$). The di-deprotonated form $^{(3,10)}\mathbf{3}^{2-}$ was found to be the most stabilized one ($\Delta H_f = -1464\,\mathrm{kJ \cdot mol^{-1}}$) as compared to all other di-deprotonation site combination species. Its heat of formation was thus found to be of about the same value as that of the mono-deprotonated system. These findings were nicely in accord with the two highly acidic *bay*-hydroxyl groups ($pK_a = 1.4$ and 3.3) of **3** found experimentally [9].

Upon loss of an electron, $^{(3,10)}\mathbf{3}^{2-}$ becomes the most stable radical anion $^{(3,4)}\mathbf{3}^{\cdot-}$ ($\Delta H_f = -1317\,\mathrm{kJ \cdot mol^{-1}}$) as compared to all other possible charge/radical combinations. As has been found for such a radical species formed from an anion in the case of **2** [7], a *Mulliken* population analysis of $^{(3,4)}\mathbf{3}^{\cdot-}$ revealed that there is

no zwitterionic form present as discussed first-hand [1, 6], but the electron density was uniformly distributed over the molecular frame. This radical anion species $^{(3,4)}\mathbf{3}^{\cdot -}$ is now easily and of all other possibilities best stabilized by loosing another proton in position 10 affording $^{(3,4,10)}\mathbf{3}^{\cdot 2-}$ ($\Delta H_f = -1326\,\mathrm{kJ \cdot mol^{-1}}$). Accordingly, the radical ion generated by loss of an electron from the dianion is itself highly acidic and can intermolecularly transfer its proton to the indicator system. This result is in agreement with the result obtained for the similar process in **2** [7]. Obviously, the doubly *bay*-deprotonated fringelite $\mathbf{3}^{2-}$ behaves similar with respect to deprotonation as the mono-*bay*-deprotonated hypericin $\mathbf{2}^{-}$.

Calculations on the natural product stentorin (**1**) led to results comparable to those for its parent system **3**. However, due to its lower symmetry, which is reduced by the two isopropyl groups in positions 2 and 5, most species resulting from its deprotonation and electron-loss became non-degenerate with rather small energy gaps between them. Nevertheless, a similar conclusion as in **3** could be reached. The 7,14-dioxotautomer propeller species of **1** proved to be the most stable one ($\Delta H_f = -1303\,\mathrm{kJ \cdot mol^{-1}}$). Mono-deprotonation resulted in the most stable $^{(3)}\mathbf{1}^{-}$ *bay*-phenolate ($\Delta H_f = -1571.8\,\mathrm{kJ \cdot mol^{-1}}$), which as an example of the extent of non-degeneracy might be compared to the only slightly less stable $^{(10)}\mathbf{1}^{-}$ *bay*-phenolate ($\Delta H_f = -1571.7\,\mathrm{kJ \cdot mol^{-1}}$). The $^{(3,10)}\mathbf{1}^{2-}$ species proved to be the most stable di-deprotonated system ($\Delta H_f = -1573\,\mathrm{kJ \cdot mol^{-1}}$). Upon loss of an electron, the most stable system out of all other di-anion charge combinations was found to be the $^{(3,4)}\mathbf{1}^{\cdot -}$ species ($\Delta H_f = -1429\,\mathrm{kJ \cdot mol^{-1}}$). The latter was then found to be highly acidic due to a significantly stabilized $^{(3,4,10)}\mathbf{3}^{\cdot 2-}$ species ($\Delta H_f = -1441\,\mathrm{kJ \cdot mol^{-1}}$) and could thus easily donate intermolecularly a proton to the indicator dye.

In conclusion, the experimental study using two-photon excitation fluorescence spectroscopy together with the results of semiempirical calculations nicely document the pronounced acidification of the photosensory pigments of the fringelite D type parent chromophoric system upon excitation and electron transfer. Intermolecular proton transfer following excitation and electron loss of the pigment thus seems to be a favorable candidate for the primary step in the signal transduction cascade of the photosensory pigments of the stentorin and blepharismin type.

## Experimental

Hypericin (**2**) [15] and fringelite D (**3**) [9] were prepared as described recently; the indicator **4** was of commercial origin (Fluka). To 1 $cm^3$ of a solution (10 µmol/$cm^3$) of dipalmitoylphosphatidylcholine (Sigma) in $CHCl_3$ (p.A. Merck, percolated over basic $Al_2O_3$ before use), 2 $cm^3$ of a 0.95 µmol/$cm^3$ solution of **2** or **3** in methanol were added, and the solvent was evaporated in a slow stream of $N_2$. The residue was again dissolved in 1 $cm^3$ $CHCl_3$ and dried once more by means of a stream of $N_2$. This procedure was repeated twice. Finally, the residual solvent was removed by evaporation at $10^{-3}$ mm Hg for 15 min. The resulting film was mixed with 100 $mm^3$ of a solution of 1 g/$dm^3$ (1.92 µmol/$cm^3$) **4** in 30 m*M* $NaN_3$/120 m*M* NaCl buffer ($pH \approx 7.1$). This heterogeneous mixture was then sonicated (Elma, Transsonic T 460) for 5 min at 56–57°C, resulting in the vesicle solution. Vesicles prepared by means of this procedure have been shown to consist of a double membrane encapsulating a $H_2O$ phase of about 12 nm diameter and an outer diameter of about 20 nm [16]. The vesicle solution was then applied to the sample holder of the epifluorescence microscope.

The epifluorescence experimental setup is illustrated in Fig. 1. The sample (4) was illuminated for 10 ms by 0.5–18 mW of 850 nm light from a frequency doubled Nd-YAG pumped femtosecond

Ti:sapphire laser (1, Millenia/Tsunami, Spectra Physics, Mountain View, USA). At the laser output the pulse train was characterized by a pulse width of 150 fs and a repetition rate of 80 MHz. The size of the excitation volume was given by measuring 0.13 $\mu m^2$ in lateral and 1 μm in axial direction. The sample fluorescence was collected using a ×100 objective (Plan-Neofluar, NA = 1.3, Zeiss, Jena, D) in an epifluorescence microscope (Axio Vert S100TV, Zeiss, Jena, D). The emitted fluorescence was first separated from scattered light by filters 5 (a costume made dichroic 725 dcsp, Chroma, Brattleboro, VT. USA, LP 700, Newport, Irvine, USA, and a BG 40, Schott, Mainz, D) and then split by the dichroic beamsplitter 7 (550DRLP02. Omega, Brattleboro, VT, USA) into two emission paths characterized by the dotted trace of Fig. 4. The resulting two fluorescence signals were detected by two photon counting avalanche diodes 8 and 9 (SPCM-AQ-141 and SPCM-AQR-131, both from EG & G, Dumberry, Canada) in combination with an interface card (PMS300, Becker & Hickl, Berlin, D). By means of the flipable mirror 6 the emission spectra could be alternatively measured using the monochromator 10 (Triax 190, Instruments SA, Cedex, F) and the photomultiplier 11 (R464, Hamamatsu Photonix K.K, Hamamatsu City, J).

The AM1 method [17] from the MOPAC package [18] was used. Calculations were performed at the SGI Origin 2000 computer of the LIZENS (*Linzer Zentrum für numerische Simulation*). For the input geometries the results of MM3 calculations [19] were adopted. It should be mentioned that after optimization of the different species a propeller conformation of the 7,14-dioxo-tautomer resulted that was virtually identical for all cases studied.

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