

BRIEF COMMUNICATION

PROTON RELEASE FROM *STENTOR* PHOTORECEPTORS IN THE EXCITED STATES

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ABSTRACT Steady-state and picosecond pulse excitations of the photophobic-phototactic receptors isolated from *Stentor coeruleus* produced anionic species predominantly in the excited singlet state, although neutral photoreceptors in the ground state were exclusively excited. The same photoreceptor in vivo also emits fluorescence from the excited state of its anionic species, with an excitation spectrum identical to the absorption spectrum of the neutral species in the ground state. The excited state dissociation of protons from the photoreceptor chromophore (stentorin; hypericin covalently linked to protein) efficiently occurs in <10 ps. A possible role of the transient-proton release from the photoreceptor, in the signal transduction photoresponse of *Stentor*, is briefly discussed.

INTRODUCTION

Stentor coeruleus exhibits photophobic and negative phototactic behavior in response to red light (1, 2). Measurements of light-induced pH changes across *Stentor* cell membranes in vivo and in model liposome systems suggest that proton release from the photoreceptor might play a primary role as an initial transduction signal following light perception by the organism (3–5). The native photoreceptor protein has been isolated and partially characterized (3, 6, 7). In the present communication, we examine efficiency of proton release from the *Stentor* photoreceptor by nanosecond and picosecond excitations.

METHODS

Stentor photoreceptor was isolated by ammonium sulfate fractionation and sucrose density gradient centrifugation, followed by isoelectric focussing ($pI = 4.1 \pm 0.1$), as previously described (6, 7). Hypericin, purchased from ICN Nutritional Biochemicals (Cleveland, Ohio), was used after purification by paper- or thin-layer chromatography. Nanosecond fluorescence lifetimes were measured on an SLM phase-modulation lifetime spectrometer (SLM Instruments, Urbana, Ill.) and two-component lifetime analyses were carried out by means of the classical de Prony method described by Weber (8).

Picosecond pulse excitation was performed with the 527-nm second harmonic of a mode-locked neodymium phosphate glass laser (pulse width ~ 10 ps) and fluorescence emission was detected by an

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Electro-Photonics Photochrom II streak camera with an S-20 photocathode and PAR multichannel analyzer, as described elsewhere (9). Coaxial detection geometry was used and polarization bias was eliminated by using an analyzing polarizer set at 54.7° to the polarization direction of the exciting pulse. Fluorescence emission was detected through the use of appropriate Schott glass cut-off filters (Schott, Inc., New York) and Ditic wide band interference filters (Ditic Optics, Inc., Marlboro, Mass.). Data were collected and analyzed on a PDP11/34 minicomputer. Analysis was performed with a nonlinear least-squares program using a Marquardt algorithm for both the single and double exponential decays. Plots were output on an HP 7221 A plotter. The relative time shift between excitation pulse and emission was a parameter of the fit.

RESULTS AND DISCUSSION

The native *Stentor* photoreceptor in vivo and in vitro emits fluorescence (λ_F 660 nm) predominantly from the anionic species, although its absorption and fluorescence excitation spectra are those of the neutral species (λ_F 610 nm). The photoreceptor proteins isolated from *S. coeruleus* also exhibit a predominantly anionic fluorescence (660 nm), along with a decrease in the fluorescence quantum yield of the neutral species, if the photoreceptor protein molecules are "reconstituted" at higher concentrations (Fig. 1).¹ However, hypericin in methanol and in 10 mM Tris buffer, pH 7.4, 0.1% sodium cholate, and *Stentor* photoreceptors solubilized in acetone and denatured emit fluorescence largely from the neutral species (3, 6). These results suggest that only the native photoreceptor ejects protons efficiently during its excited state lifetime, with an apparent excited state pK^* of 2.5 for the hydroxyl groups of the photoreceptor chromophore.

To elucidate the mechanism of proton release from the native *Stentor* photoreceptor, Stern-Volmer quenching of the fluorescence was examined (Fig. 2). It can be seen that fluorescence of the native photoreceptor is not quenched by the heavy atom salt, KI, although the fluorescence of hypericin and sodium cholate detergent-treated and acetone-solubilized (data not shown) photoreceptor proteins are effectively quenched by KI. Attempts to quench the native photoreceptor fluorescence with CsCl and acrylamide also failed with concentrations up to 4 M. It has been reported that the hydroxyl groups of stentorin chromophore cannot be titrated unless their proteins have been denatured (3). These observations indicate that the chromophore of a photoreceptor is buried well within the hydrophobic core of the protein, as in the photoreceptor proteins of marine dinoflagellates (10).

How, then, do protons dissociate so efficiently from the chromophore buried inside the native protein core (1 chromophore/16 kD) during the short excited state lifetime? To answer this question, we used fluorescence rise and decay methods to elucidate the excited state dissociation process in the *Stentor* photoreceptor. Fluorescence lifetimes of the anionic species of hypericin and stentorin were considerably shorter than those of the neutral species. Live *Stentor* emitted fluorescence of a 1.21- (anionic component; 90%) and 4.95-ns (neutral component; 10%) lifetimes. A 20- μ M solution of *Stentor* photoreceptor showed two component lifetimes, 1.47 (neutral; 82%) and 4.48 ns (neutral; 18%). These data are thus consistent with the steady-state fluorescence spectra shown in Fig. 1. A 30- μ M photoreceptor solution showed essentially single exponential fluorescence due to the anionic species.

Fig. 3 represents the fluorescence rise and decay of *Stentor* photoreceptor (20 μ M) at

¹The aggregates formed at 29 μ M have an apparent mol wt \geq 250,000(6).

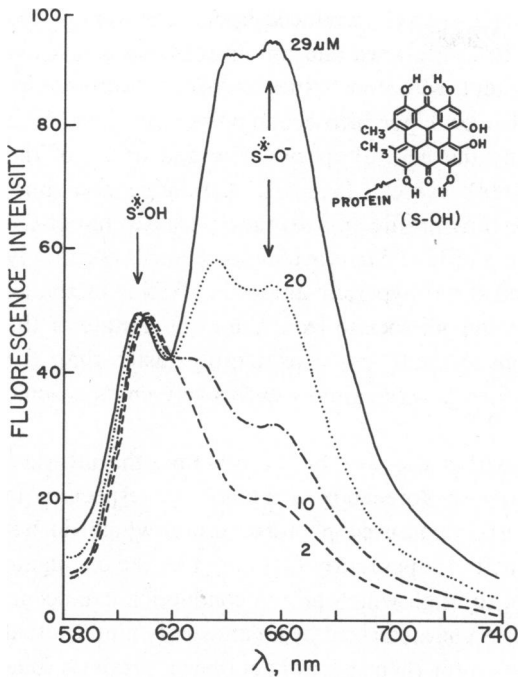


FIGURE 1

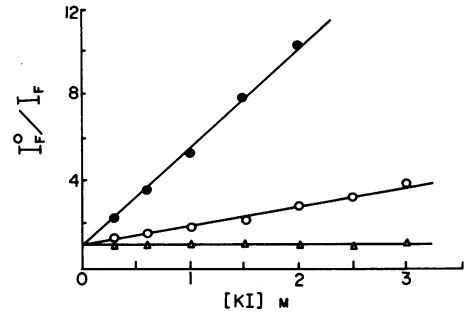


FIGURE 2

FIGURE 1 Fluorescence emission spectra of *Stentor* photoreceptor in 10 mM phosphate buffer, pH 7.4 at 295 K. The exciting wavelength was 560 nm (14 nm bp). The emission slit was varied between 6 and 7 nm to normalize intensity with respect to the 610-nm maximum (fluorescence of neutral species; S-OH*). The inset shows the structure of hypericin; the nature and site of linkage to protein are not known.

FIGURE 2 Stern-Volmer quenching of the photoreceptor fluorescence by KI in 10 mM Tris buffer, pH 7.4 at 295 K. (●): free chromophore (hypericin) in 0.1% sodium cholate detergent, $k_q = 9.1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. (○): *Stentor* photoreceptor in 0.1% sodium cholate detergent, $k_q = 2.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. (△) *Stentor* photoreceptor, pH 7.4; $k_q = 0$. Ionic strengths of KI solutions were kept constant with NaCl.

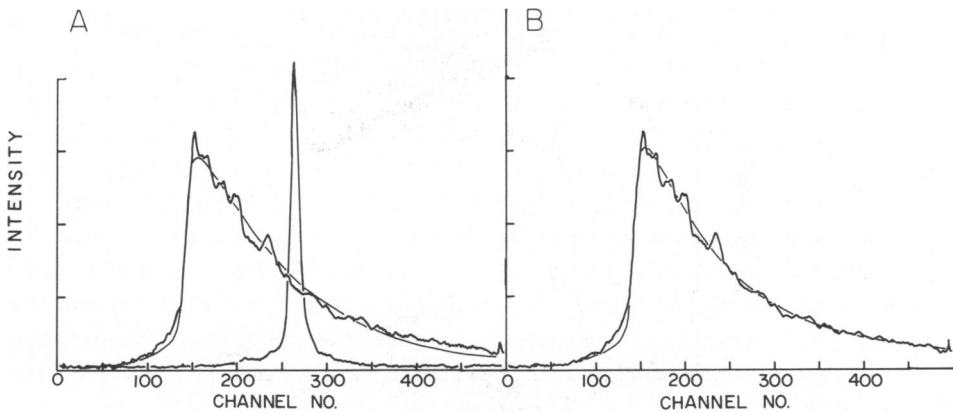


FIGURE 3 Fluorescence rise and decay of *Stentor* photoreceptor (20 μM) in 10 mM phosphate buffer, pH 7.4 at 291 K, excited with picosecond pulse (typical intensity profile is shown in panel A). (A) single exponential fit (thin line) with decay time of 1.94 ns and χ^2 of 7.4. (B) two-exponential fit (bold line) with $\tau_1 = 1.35 \text{ ns}$ (81%) and $\tau_2 = 4.96 \text{ ns}$ (19%). Time scale: 19.22 ps/channel.

660–680 nm, where fluorescence from the anionic species, produced upon excitation of the neutral photoreceptor, predominates (Fig. 1). Both lifetimes and amplitudes of the neutral and anionic fluorescences are in good agreement with the values obtained from phase-modulation measurements, within experimental error. Upon picosecond pulse excitation of the photoreceptor at 527 nm, which is absorbed by the neutral species, rise and decay of the anionic fluorescence can be followed on a streak camera (Fig. 3). A reliable rise time, however, could not be resolved, because the rise-time profile approximately traced that of the excitation pulse (Fig. 3 A). In fact, the rise-time profile of *Stentor* photoreceptor is essentially identical with that of the fluorescence of (ground state) hypericin anions in alkaline methanol solution (Fig. 4). These results suggest that protons dissociate from the excited state of the neutral chromophore at a rate (with time constant ≤ 10 ps) considerably faster than the diffusion-controlled rate. It is noteworthy that live *Stentor* almost exclusively emits anionic fluorescence (*vide supra*; reference 4).

Since the chromophores are deeply buried within the core of the proteins, the ultrafast release of protons from the excited state of native photoreceptor is remarkable, especially in comparison to free chromophore (hypericin) and denatured photoreceptors, which do not exhibit efficient proton release. Fast proton transfer is apparently facilitated by the conjugate acid-base network of the photoreceptor protein, through which proton conduction can occur. Thus, it is significant that *Stentor* photoreceptor is characterized by an unusually high content of both aspartate and glutamate, which are known for their general acid-base, catalytic roles in many enzyme systems. It is indeed possible to show that protons are ejected from liposome-enclosed photoreceptors upon irradiation with red light (4, 5). Ultrafast proton transfer with a rate constant ($2 \times 10^{12} \text{ M}^{-1}\text{s}^{-1}$) greater than the diffusion-controlled rate has also been observed in the excited state of pyrene 1-carboxylic acid embedded in the hydrophobic phase of reverse micelle, dodecylammonium propionate (11).

It is possible that a light-induced proton flux, generated via excitation of the imbedded chromophore in *Stentor* photoreceptor, represents an initial event in sensory signal transduction, which subsequently triggers action potentials (Ca^{2+} flux?) effecting ciliary reversal in *S. coeruleus*. In order for the *Stentor* photoreceptor to be an effective signal transducer,

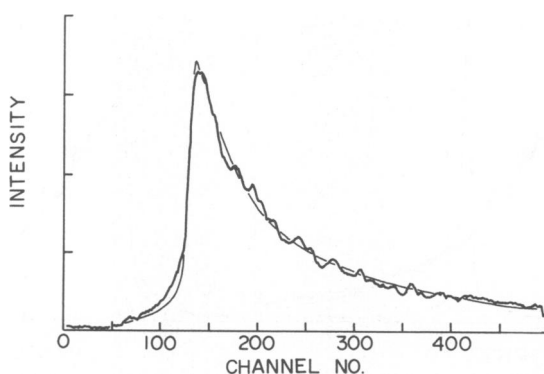


FIGURE 4 Fluorescence rise and decay of hypericin ($20 \mu\text{M}$) in 0.1 M KOH methanol at 291 K . Conditions for measurements were the same as in Fig. 3. Two-exponential fit (thin line), $\tau_1 = 0.56 \text{ ns}$ (68%) and $\tau_2 = 3.66 \text{ ns}$ (32%). Time scale: 19.22 ps/channel .

ultrafast proton release must be coupled with a conformational change of the protein, which slows down the reassociation of dissociated protons. Work is in progress to elucidate such light-induced conformation changes and the resulting light intensity-dependence of the fluorescence in the *Stentor* photoreceptor.

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REFERENCES

1. Song, P. S., D. Häder, and K. L. Poff. 1980. Step-up photophobic response in the ciliate, *Stentor coeruleus*. *Arch. Microbiol.* 126:181.
2. Song, P. S., D. Häder, and K. L. Poff. 1980. Phototactic orientation by the ciliate, *Stentor coeruleus*. *Photochem. Photobiol.* 32:781.
3. Walker, E. B., T. Y. Lee, and P. S. Song. 1979. Spectroscopic characterization of the *Stentor* photoreceptor. *Biochim. Biophys. Acta.* 587:129.
4. Walker, E. B., M. Yoon, and P. S. Song. 1981. The pH dependence of photosensory responses in *Stentor coeruleus* and model systems. *Biochim. Biophys. Acta.* 634:289.
5. Song, P. S. 1981. Mechanisms of photosensory transduction in *Stentor coeruleus*. *Biochim. Biophys. Acta.* In press.
6. Walker, E. B. 1980. Photosensory transduction in *Stentor coeruleus*. Ph.D. Dissertation. Texas Tech University, Lubbock, Tex. 1-214.
7. Song, P. S., E. B. Walker, and M. Yoon. 1980. Molecular aspects of photoreceptor function in *Stentor coeruleus*. In *Photoreception and Sensory Transduction in Aneural Organisms*. F. Lenci and G. Colombetti, editors. Plenum Publishing Corp., New York. 241-252.
8. Weber, G. 1980. Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements. Proceedings of the NATO School on Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology, St. Andrews, Great Britain, March 1980. 1-22.
9. Robinson, G. W., T. A., Caughey, and R. A. Auerbach. 1978. Picosecond emission spectroscopy with an ultraviolet sensitive streak camera. In *Advanced Laser Chemistry*. A. H. Zewail, editor. Springer-Verlag, Inc., New York. 108-125.
10. Prasad, K., and P. S. Song. 1977. The chromophore topography and binding environment of peridinin-chlorophyll *a*-protein complexes from marine dinoflagellates. *Biochim. Biophys. Acta.* 495:220.
11. Escabi-Perez, J. R., and J. H. Fendler. 1978. Ultrafast excited-state proton transfer in reversed micelles. *J. Am. Chem. Soc.* 100:2234.