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pH-sensitive fluorescent dye as probe for proton uptake in photosynthetic reaction centers☆

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Abstract

Isolated and purified reaction centers (RC) from *Rhodobacter sphaeroides* R-26.1 were solubilised in detergent with excess quinone and external electron donors and illuminated in the presence of pyranine. The pH change accompanying the reaction center photocycle was monitored by recording the variation of the pyranine fluorescence intensity. Using Q_B-depleted reaction centers or blocking the photocycle with terbutryne strongly reduced the pH change. The usefulness and limits of this technique in monitoring the pH changes during the RC photocycle are also discussed.

Keywords: Fluorescent dye; *Rhodobacter sphaeroides*; Pyranine; Proton uptake

1. Introduction

The photosynthetic reaction center (RC) of purple bacteria is an integral membrane enzyme that, upon light absorption and in the presence of exogenous electron donors, undergoes a photocycle resulting in the double reduction and double protonation of the loosely bound lipophilic electron carrier ubiquinone-10. The light-generated ubiquinol leaves the RC and reaches a second redox enzyme (i.e. ubiquinol/cytochrome oxido-reductase) where is oxidised and releases the protons, generating the transmembrane proton-gradient required for the metabolic activities of the cell [1].

In isolated RCs, the photocycle can be restored in the presence of an external electron donor (i.e. cytochrome *c* or ferrocene) and a pool of quinone molecules that exchange with the quinone bound to the Q_B-binding site of the protein. The enzyme catalytic role can be summarised by the following equation:

$$2\text{Cyt } c^{2+} + \text{UQ}_{10} + 2\text{H}^+ \xrightarrow{\text{RC}} 2\text{Cyt } c^{3+} + \text{UQ}_{10}\text{H}_2. \quad (1)$$

Being UQ_{10} a highly hydrophobic molecule, upon illumination protons will be taken up from the solution to form the quinol, ending up sequestrated into the micellar phase. In an unbuffered solution, the increase of the pH accompanying the photocycle can be easily followed by pyranine, a pH-sensitive fluorescent dye [2].

This paper represents a preliminary study for employing the fluorescence technique to monitor the RC photocycle.

2. Experimental

Reaction centers were isolated from *Rhodobacter sphaeroides* strain R-26.1 following the procedure described by Isaacson et al. [3]. Protein purity was checked using the ratio of the absorbances at 280 and 802 nm (A_{280}/A_{802}), which was kept below 1.3, and the ratio of the absorbances at 760 and 865 nm (A_{760}/A_{865}), which was kept equal to or lower than 1.

☆ Dedicated to the memory of Prof. Mario Della Monica.

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The average quinone content of the preparation was (Q_{10}/RC) = 1.8. The Q_B-binding site depletion was accomplished following the procedure of Okamura et al. [4] and the final preparations had a quinone content (Q_{10}/RC) = 1.05 ± 0.05 determined by the charge recombination.

Charge recombination kinetics were recorded at 865 nm using a kinetic spectrophotometer of local design implemented with an Hamamatsu R928 photomultiplier and a Nd-Yag Laser (Quanta System) used for RC photo-excitation. The laser flashes were saturating. Data were collected onto a Digital Oscilloscope (Tektronix TDS 3052) and trace deconvolution was performed using a C-code developed in our lab or commercial softwares (Sigmaplot) based on the Levenberg-Marquart non-linear fitting. The decay traces were recorded up to near ($\geq$ 98%) complete recovery.

Fluorescence experiments were performed in Triton X-100 (TX-100); the detergent was exchanged by gel exclusion chromatography with the following procedure: 50 μl of 50–60 μM LDAO-solubilised RCs were diluted in 0.5 ml of an unbuffered solution containing 0.06% TX-100 and 100 mM KCl. This solution was loaded onto a Sephadex G-50 column previously equilibrated with the same solution. The RC concentration for the fluorescence experiments was adjusted to 1–2 μM. The solution containing the Triton-RC complex is added with excess ferrocyanide. The final concentrations of ferrocene, pyranine and UQ-10 were 50, 15 and 60 μM, respectively. Similar conditions were employed by Osvath et al. [5] for monitoring the surface and bulk pH changes associated to RC single turnover with a pH-sensitive dye. The samples were kept in the dark. The

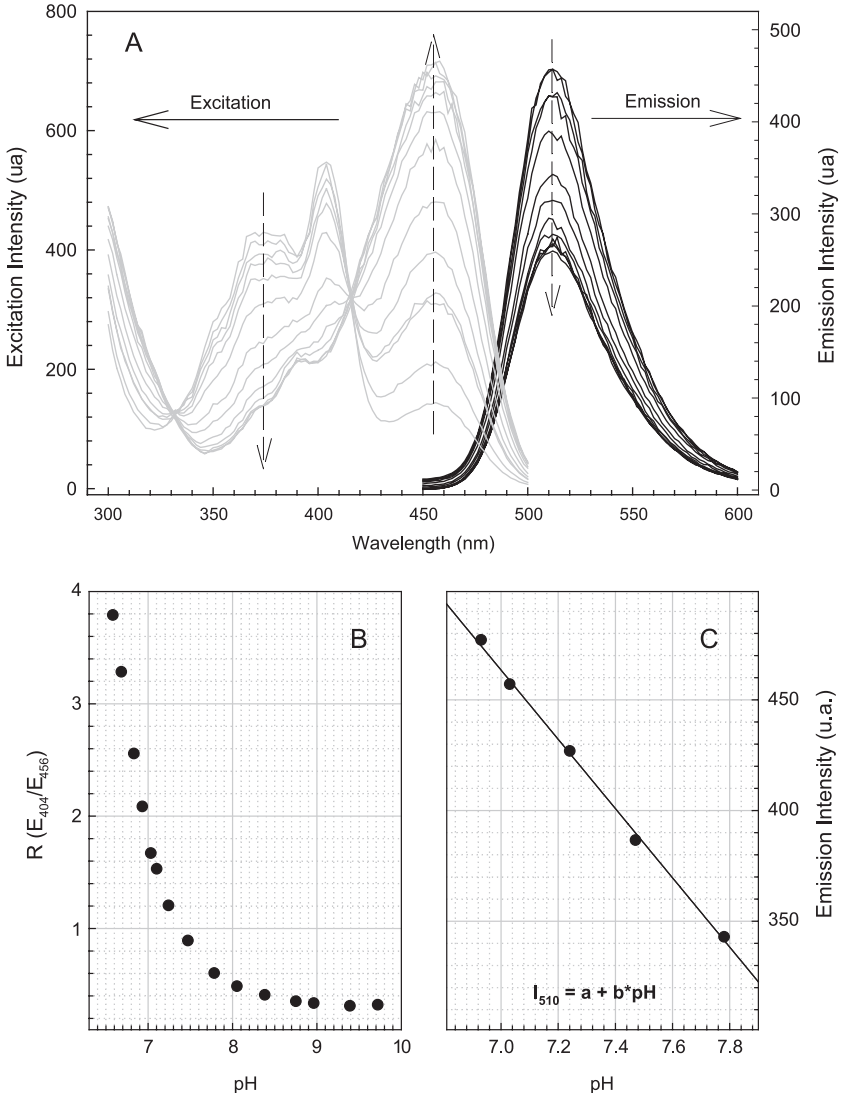

Fig. 1. (A) Excitation (gray line) and emission (black line) spectra of pyranine as function of pH recorded in phosphate buffer. The dashed arrows indicate the direction of pH increase. Excitation spectra were recorded by monitoring the fluorescence at 510 nm. Emission spectra are obtained with excitation at 410 nm with slits at 2.5 nm. (B) Calibration of the pH dependence of the intensity ratio *R* of the two peaks (404 and 456 nm) in the excitation spectra. This value is used to infer the initial pH of the solution. (C) Linear pH dependence of the fluorescence intensity in the pH range 6.9–7.8. Best fit parameters: $a = 1566 \pm 30$ and $b = 157 \pm 4$.

fluorescence spectra and the changes in the fluorescence intensity were recorded with a Cary Eclipse Fluorescence spectrophotometer (Varian). Illumination of the sample via an optical fiber (diameter 0.9 cm) was obtained by 150 W Oriel incandescent lamp.

Pyranine (trisodium 8-hydroxypyrene-1,3,6-trisulfonate), ferrocene, ferrocyanide, TX-100 and ubiquinone-10 were purchased from Sigma.

The pH of the solution was measured by a freshly and carefully calibrated glass electrode. The calibration experiments of Fig. 1B and C was performed in solution buffered with phosphate buffer and the pH was adjusted with HCl or NaOH.

3. Results and discussion

Pyranine is a water-soluble fluorescent dye which is very sensitive to pH in the interval close to the pK_a = 7.2 of the ionisation of the hydroxyl group of the molecule [2]. Excitation and fluorescence spectra of the aqueous solutions of the dye are shown in Fig. 1A. The excitation spectra show two isosbestic points (at 331 and 416 nm) clearly indicating the inter-conversion of two absorbing species with the two maxima at 404 and 456 nm, respectively. The ratio between the excitation peaks at 404 and 456 nm (Fig. 1B) can be calibrated and used as pH-indicator allowing to estimate the initial and final pH values of the solution in the experiment without the use of the pH glass electrode. In the pH interval close to the pyranine pK_a, the linear dependence of the emission intensity upon the pH (Fig. 1C) allows to directly calculate the number of protons that have been uptaken from or released to the solution during the illumination.

To follow the pH changes induced by the photocycle, the experiments must be performed in the presence of a large quinone pool and in a solution with poor buffer capacity. This conditions are satisfied by reducing the concentration of the buffer (≤ 10 μM) and by exchanging the detergent

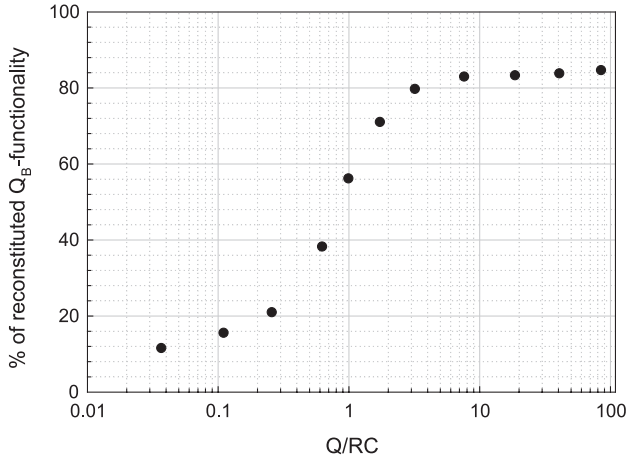

Fig. 2. Titration curve of Q_B-depleted RCs solubilised in 0.06% TX-100. The Q_B-functionality is reconstituted up to 85% from a Q/RC of 8–10.

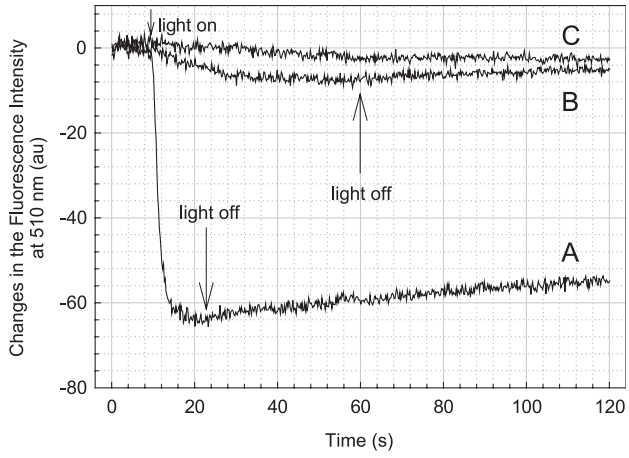

Fig. 3. Time course of the fluorescence changes of a RC solution upon illumination. Experimental conditions: 0.06% TX-100, 1.2 μM RC, 50 μM ferrocene, 15 μM pyranine, pH = 6.92. Before the experiment, the fluorescence readout was set to zero. Trace A is recorded in the presence of excess (60 μM) quinone solubilised in TX-100; trace B as in trace A in the presence of excess terbutryne; trace C as in trace A but with Q_B-depleted RCs (Q/RC = 1.05 ± 0.05).

LDAO used for the isolation and purification procedure with TX-100. This latter detergent has a rather small buffer capacity $\beta \sim 0.1$ (mM_{H^+}/pH) that remains constant in the pH range 5.5–7.4, compared to the LDAO case for which the buffer capacity is larger and increase from $\beta \sim 0.2$ mM_{H^+}/pH at pH 7.5 to $\beta \sim 1.5$ mM_{H^+}/pH at pH 5.5 [6]. Furthermore, TX-100 in NaCl forms micelles [7,8] with an aggregation number of 140 molecules per micelle, larger than the LDAO one that has an aggregation number of 70. Larger micelles accommodate a larger pool of quinone although working above the critical micelle concentration (cmc) induces the formation of TX-100 micelles not in contact with RC so that not all of the added quinones may be available for the photocycle. This issue was addressed by checking the functional reconstitution of the Q_B-binding site in Q_B-depleted RCs solubilised in 0.06% TX-100. The actual reconstitution of the Q_B-functionality is shown in Fig. 2 and is found in the order of 85% under the condition used for the fluorescence experiments.

A typical experiment of pH change associated to the photocycle is shown in Fig. 3 where the light induced pyranine fluorescence change is recorded as function of time on a minutes time scale. Upon illumination of a RC in TX-100 solution (trace A), an initial sharp fluorescence decrease is followed by a plateau reached within 5–10 s. The fluorescence intensity remains constant as long as the sample remains illuminated. Once light is turned off the fluorescence slowly increases probably because of the ubiquinol oxidation by the atmospheric oxygen that releases protons to the solution decreasing the pH. Trace B shows an experiment under the same conditions as above but in the presence of the herbicide terbutryne, a known inhibitor of the RC photocycle that binds to the Q_B-pocket of the enzyme. The inhibitory effect is clearly shown by the

dramatic decrease in the rate of proton uptake and by the level of the plateau reached after 40–50 s of sample illumination. Notwithstanding, the high terbutryne affinity for the RC with an dissociation constant in the order of μM [9–11], even with the highest obtainable inhibitor concentration in TX-100 a photocycle inhibition higher than 85% was not obtained. From this respect, not only the value of the constant but also the rates of binding/unbinding of the inhibitor, quinone and semiquinone should be taken into consideration. Once the light is turned off, the fluorescence intensity tends to return to the initial value. In trace C is shown, for comparison, the light induced fluorescence changes that accompanies the photocycle of a Q_B-depleted RC solution. The extremely slow rate of the fluorescence change and the very small plateau recorded under this condition are probably due to the proton uptake associated to the radical anion Q_A^- formation and to the residual occupancy of the Q_B pocket. In trace C, the light was kept on for the entire duration of the experiment.

In this paper, we have shown that a pH-sensitive fluorescent dye can be used to monitor the proton uptake associated to the RC photocycle. This experimental approach is rather simple and the use of the fluorescent technique makes it also quite sensitive. A few points, though, must be considered in planning these experiments: (a) the use of pyranine imposes a restricted pH range close to the pK_a of the dye ($6.8 < pH < 7.8$). Other pH interval must be studied with different hydrophilic fluorescent dyes and (b) the intrinsic buffer capacity of the RC [6] varies from 0 to 100 $\mu M_{H^+}/pH/\mu M_{RC}$ passing from $pH = 8$ to $pH = 6.2$. Such value must be taken into account when performing the experiments in unbuffered solutions. Helpful can also be keeping the enzyme concentration as low as possible.

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