A Correlation between pH and Fluorescence Lifetime of 2′,7′-Bis(2-Carboxyethyl)-5(6)-carboxyfluorescein (BCECF) In Vivo and In Vitro

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The pH dependence of the fluorescence lifetime of BCECF both in solution and in intact cells of *Halobacterium salinarum* has been measured with time-resolved fluorescence spectroscopy. A correlation between pH and fluorescence lifetime in vivo is different from that in vitro. The difference is ascribed to the local field produced by some protein membrane in a biological system.

Measurements of intracellular pH provide valuable information on physiological states of cells. pH-Sensitive fluorescent dyes have generally been used to determine intracellular pH.[1–8] Either excitation ratio or emission ratio methods have been adopted for quantification of the fluorescence intensity because of the ambiguities of fluorescence intensities.[3–6] Fluorescence microscopy with pH-sensitive dyes offers a method to determine pH with spatial resolution; however, the ratio methods are difficult to be combined with a confocal microscope because it is not easy to retain a constant focal depth of different excitation wavelengths and because the emission ratio imaging is complicated owing to wavelength-dependent absorption. Fluorescence lifetime imaging (FLIM) can solve these problems.[7–11] Fluorescence lifetime is independent of photobleaching, excitation power, or other factors that limit intensity measurements. Neither of the ratio methods are necessary for the lifetime-imaging measurements, so that the FLIM method is compatible with a confocal microscope.[8–11]

2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) is one of the most widely used fluorescent dyes for evaluation of intracellular pH.[1,2,5–8] Its fluorescence intensity depends on pH because of the pH-dependent ionic equilibria. The acetoxymethyl (AM) ester of BCECF, i.e., BCECF/AM, is membrane-permeable, and cells can be loaded with dyes. Then, the AM ester is cleaved by intracellular esterase, and the generated BCECF remains inside and is available for the indicator of intracellular pH. In application of the FLIM method to the evaluation of intracellular pH, the correlation between pH and fluorescence lifetime is significant. Several groups have estimated the pH dependence of the fluorescence lifetime of BCECF in solution using phase-modulated fluorometry.[7,8] As far as we know, however, no one has reported the direct measurements of the correlation between the fluorescence lifetime of BCECF and intracellular pH by time-resolved fluorescence techniques. In the present study, we have measured the fluorescence decay of BCECF in solution and in *Halobacterium (Hb.) salinarum*[5,6] at different pH, and the correlation between pH and the fluorescence lifetime of BCECF is examined under both the conditions.

BCECF and BCECF/AM were purchased from Dojindo Co. Aqueous solutions of BCECF were prepared in 1.0×10^{-2} mol dm^{-3} 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer. The concentration of BCECF, chemical structure of which is shown in Figure 1A, was 1×10^{-5} mol dm^{-3}. The strains of *Hb. salinarum*, S9, were cultured in peptone medium at 37 °C at pH 7.0 for six days.[12] The cells were washed and resuspended by the basal salt solution (4 mol dm^{-3} NaCl containing 2.5×10^{-2} mol dm^{-3} HEPES) at pH 6.8. A 10-cm^3 cell suspension was incubated with a 10 mm^3 of DMSO solution of BCECF/AM at 1.0×10^{-2} mol dm^{-3} in the dark at 18 °C for three days. The cell suspension was washed out with the basal salt solution repeatedly until the supernatant showed no fluorescence.[5,6] Then, the dye-loaded cells were resuspended in the basal salt solution.

We carried out fluorescence decay measurements using a single-photon counting method.[13] The second harmonic of the output from a mode-locked Ti:sapphire laser (Spectra Physics, Tsunami, pulse duration 80 fs) was used for excitation. Fluorescence from the sample was dispersed and detected by a microchannel-plate photomultiplier (Hamamatsu, R3809U-52).

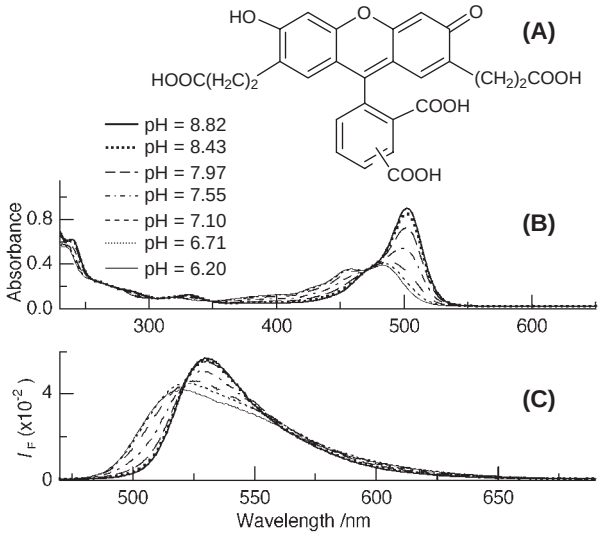

Figure 1. Chemical structure of BCECF (A). Absorption (B) and fluorescence (C) spectra of BCECF in HEPES buffer for different values of pH. Excitation wavelength was 450 nm.

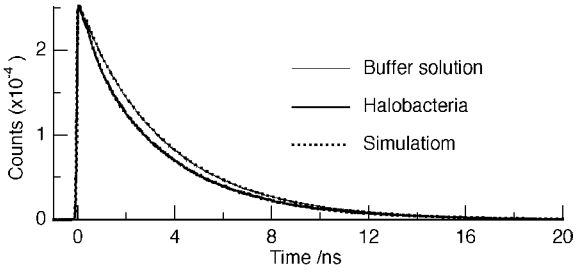

Figure 2. Fluorescence decay of BCECF in HEPES buffer at pH 7.6 and in *Hb. salinarum*. Excitation and fluorescence wavelengths were 450 and 530 nm, respectively.

The instrumental response function had a pulse width of ca. 60 ps (FWHM).

Figures 1B and 1C shows the absorption and fluorescence pectra of BCECF in solution, respectively, for different values of pH. The pH-dependent behavior of BCECF in absorption as well as in emission spectra is the same as that of the parent fluorescein.[14] The absorption bands with a peak at 452 and 502 nm, respectively, are assigned to the monoanion and dianion species of the fluorescein chromophore, and the molar ratio of the dianion increases, as solution pH increases. The dianion exhibits strong fluorescence with a peak at 530 nm, which can be used as a pH indicator.[14] The fluorescence spectrum of the monoanion is relatively broad in the 500–600-nm region.

We have observed the pH dependence of the fluorescence lifetime of BCECF in aqueous solution using time-resolved fluorescence spectroscopy. The fluorescence decay observed in solution is shown in Figure 2. The decay curves in solutions are fitted by assuming a biexponential decay, i.e., $\Sigma A_i \exp(-t/\tau_i)$, where A_i and τ_i denote the pre-exponential factor and lifetime of component i (= 1, 2), respectively. Plots of the solution pH against the average fluorescence lifetime (τ_f) given by $\Sigma A_i \tau_i$ are shown in Figure 3. The correlation between pH and the fluorescence lifetime in solution is almost the same as that obtained with the frequency-domain method.[7,8] The correlation is well fitted by the following polynomial function of pH in the range from 5.6 to 8.4 (Figure 3):

$$\mathrm{pH} = -514.51 + 483.09 \times \tau_f - 149.38 \times \tau_f^2 + 15.434 \times \tau_f^3 \quad (1)$$

The polynomial behavior of the correlation function may result from the pH dependence of the molar ratio between the monoanionic and dianionic species.

Figure 2 also shows the fluorescence decay of BCECF observed in *Hb. salinarum*. A triexponential decay was assumed to fit the decay observed in vivo. The pH dependence of the fluorescence lifetime in *Hb. salinarum* could be measured using monensin, which is a kind of Na^+/H^+ ionophore and makes an equilibrium between intracellular and extracellular pH.[5,6] Thus, the cell suspension was mixed with a 2.5 mm^3 of DMSO solution of monensin at 1.0×10^{-2} mol dm^{-3}. After 10 min, pH of the suspension was adjusted to give different values of pH from each other, at each of which the fluorescence decay was measured.

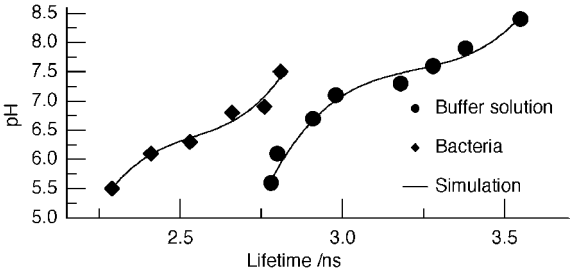

Figure 3. Plots of pH against the fluorescence lifetime of BCECF in HEPES buffer (●) and in *Hb. salinarum* (◆). Excitation and fluorescence wavelengths were 450 and 530 nm, respectively.

Plots of the intracellular pH against τ_f of BCECF are shown in Figure 3. The correlation function between the intracellular pH and τ_f is found to be different from that in solution. This indicates that substantial consideration must be paid to calibrate intracellular pH using solution data.[8] The average fluorescence lifetime is shorter in vivo than in solution even at the same pH, which may be ascribed to the local field produced by some protein membrane that affects the chromophore in vivo,[13,15] though the difference in refractive index of the matrix may be also considered.[10] The correlation function of the intracellular pH in the range from 5.5 to 7.5 is given as follows:

$$\mathrm{pH} = -440.42 + 523.91 \times \tau_f - 205.49 \times \tau_f^2 + 26.961 \times \tau_f^3 \quad (2)$$

From eq 2, we can evaluate pH of *Hb. salinarum* without ratio methods. The fluorescence lifetime in *Hb. salinarum* without monensin is evaluated to be 2.76 ns. The intracellular pH is then calculated to be 7.1, which is in reasonable agreement with that obtained from the excitation ratio method.[5]

In conclusion, the intracellular pH of *Hb. salinarum*, which is important to understand the ion transport and bioenergetics, can be evaluated using the fluorescence lifetime of BCECF. It is also suggested that the local field produced by membranes significantly affects the fluorescence lifetime of chromophore. We are now in progress to measure the spatial distribution of pH of *Hb. salinarum* using the FLIM method.[11]

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