Journal of Fluorescence, Vol. 2, No. 2, 1992

pH-Dependent Intracellular Quenching of the Indicator Carboxy-SNARF-1

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Received June 22, 1992; accepted July 9, 1992

Carboxy-SNARF-1 is an emission-changing, pH-sensitive probe for measurements of intracellular pH. However, the protonated and deprotonated forms of the dye interact differently with intracellular constituents, and this imposes new requirements on the calibration of the system. Whole spectra of intracellular and extracellular C.SNARF-1 were analyzed and showed (1) intracellular quenching which was significantly greater for the deprotonated form of the dye than for the protonated state and (2) a detectable change in pK_a. Importantly for avoiding damage to cells, this mathematical analysis allowed reference spectra for fully protonated and fully deprotonated dye to be obtained without a need for spectra measured at extreme values of pH. It is not known what constituent(s) of the intracellular milieu might be responsible for the changes in dye behavior in the cell. To address this question, preliminary experiments with cell-free buffers compared the pattern seen inside the cell with quenching by a protein (bovine serum albumin; BSA) or that due to ethanol. The BSA result was completely unlike the intracellular case in that the protonated form of the dye was quenched. Buffer containing ethanol, on the other hand, was able to mimic the essential features of the intracellular spectra.

KEY WORDS: Carboxy-SNARF-1; intracellular quenching; intracellular pH; pK_a.

INTRODUCTION

Fluorescent dyes which change spectral shape upon cation binding have proven to be extremely useful probes of intracellular calcium ion concentration [1] and pH [2,3]. In the case of pH, the most widely used dye at present is probably BCECF[4] [4–6], which has a pH-sensitive excitation spectrum. However, a new dye, carboxy-SNARF-1 (C.SNARF-1) [7,8], has been developed for use in systems where it is advantageous to use a fixed excitation and detect multiple emission wavelengths.

The successful use of a dye to measure intracellular pH depends on knowledge of its spectral intensities in the fully acidic and basic forms (i.e., an *in situ* calibration to produce "basis" spectral information). It also requires a correct value for the effective acid–base dissociation constant K_a inside the cell. Measurements commonly are made using the ratio of readings at only two wavelengths as long as the dye itself is the only source of signal [1]. However, in this case one has no redundant information, since two measurements are used to determine two unknowns, these being the contributions from the acid form and base form of the dye. Consequently, if one uses two wavelengths only, there would be no built-in indication of the problem that has occurred if an artifact such as absorption or fluorescence by other molecules, light scattering, etc., were to invalidate the underlying assumptions of the method.

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[4] Abbreviations used: BCECF, 2′,7′-biscarboxyethyl-5(and 6)-carboxyfluorescein; BSA, bovine serum albumin; C.SNARF-1 (Carboxy-SemiNAphthoRhodaFluor-1), 5′(and 6′)-carboxy-10-dimethylamino-3-hydroxyspiro[7H-benzo[c]xanthene-7,1′(3′H)-isobenzofuran]-3′-one; C.SNARF-AM, acetoxymethyl ester of C.SNARF-1; DMSO, dimethyl sulfoxide; EtOH, ethanol; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; HBSS/glucose-FBS, HBSS with 1 mg/ml glucose and 10% FBS; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MES, 2-[*N*-morpholino]ethanesulfonic acid; pH_e, extracellular pH; pH_i, intracellular pH.

To avoid these problems, whole spectra can be used in a least-squares calculation of the relative amounts of protonated and unprotonated dye in a sample. The extra information the spectrum provides allows us not just to learn the concentrations of the protonated and deprotonated fluorescent "species," but also to detect any shape changes in the fully protonated and deprotonated basis spectra due to the intracellular environment.

Analysis of a measured spectrum starts with the basis spectra for the fully protonated and fully deprotonated dye. The analysis then determines what linear combination of the basis spectra could provide the best fit to the sample spectrum. If the fit is accurate, then the relative amplitudes of the protonated and deprotonated contributions are proportional to their respective dye concentrations and can be used to calculate the intracellular pH. If the fit is poor, then no combination of the basis spectra can adequately explain the experimental spectrum and the pH cannot be calculated with accuracy. When poor fits have systematically been obtained in a cell system, this has usually meant that more appropriate basis spectra were required. An example of this was seen in a previous study [9] using the calcium chelator Indo-1, where the intracellular milieu gave rise to a large shift in one basis spectrum.

It has been reported that intracellular C.SNARF-1 also behaves differently from the extracellular form [10,11]. The spectral analysis reported in this paper extends that observation by making a better distinction between spectral effects and chemical effects. The former include spectral shifts and changes in intensity (quenching) in the protonated and deprotonated forms of the dye; the latter can give rise to changes in the apparent pK_a.

MATERIALS AND METHODS

Reagents. C.SNARF-1, its acetoxymethyl ester C.SNARF-AM, and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). MES, HEPES, Bicine, DMSO (grade 1), and delipidated bovine serum albumin (BSA; fraction V) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from GIBCO/BRL.

Buffered Solutions. High-potassium pH buffers (130 m*M* KCl, 30 m*M* appropriate buffer, 1 m*M* $MgCl_2$) were prepared and adjusted to pH values from 6.0 to 9.5 at 37°C. The buffers employed were MES for pH 6.0–6.5, HEPES for 7.0–7.5, and Bicine for 8.0–9.5. A second set of buffers was made containing 10 mg/ml delipidated BSA, and the pH was readjusted after addition of the BSA.

Dye-Loaded Cells. Chinese hamster ovarian carcinoma cells (OvCa) were originally obtained from Dr. George Yerganian of Boston Children's Hospital. They were grown in McCoy's 5A modified medium (Sigma) containing 10% heat-inactivated FBS. Flasks were kept in a 37°C humidified incubator in an atmosphere containing 5% CO_2. Cells were subcultured every 2–3 days by treatment with 0.25% trypsin (Sigma) in HBSS and washing. Cells for each experiment were harvested by trypsin treatment and then washed and suspended in HBSS/glucose-FBS at about 8×10^6 cells/ml. For each milliliter of cells, a 40-μg aliquot of C.SNARF-AM was used, which was dissolved in 20 μl of a 6% (w/w) solution of Pluronic F-127 in DMSO, followed by 150 μl of FBS. The dye was added to the cells and then incubated at room temperature for 15 min. Following incubation, the cells were washed, resuspended in 4 ml HBSS/glucose-FMS, and incubated at 37°C for 15 min to ensure complete intracellular hydrolysis of the dye ester. The cells were then counted, pelleted and resuspended in HBSS/glucose-FBS to a final concentration of 1×10^7/ml, and stored on ice until spectra could be taken. An aliquot of cells which received no dye was stored under the same conditions for use in obtaining background (light-scattering) spectra to subtract from measured spectra in data analysis steps. Cell viability was approximately 90%.

Adjustment of Intracellular pH. The method of Thomas *et al.* [12] was used to bring intracellular pH into equilibrium with extracellular pH. Cells were suspended in high-potassium medium (150 m*M* KCl, 1 m*M* $MgCl_2$, 20 m*M* HEPES) in the cuvette (1–2 min) and then 10 μg/ml nigericin and 5 μg/ml valinomycin were added. About 3–5 min was allowed to elapse before spectra were taken that were used in the calculations, by which time it was found that the spectral shape was stable and any further small changes could be attributed to dye leakage from the cells.

Fluorescence Spectroscopy. C.SNARF-1 spectra were obtained in a Perkin–Elmer 650-10S scanning spectrofluorometer with cuvettes thermostated at 37°C, using an excitation wavelength of 530 nm and monitoring the emission from 540–680 nm. Both monochromator slits were set to a 5-nm bandpass. For all spectra involving cells, equivalent baselines were measured with the appropriate combinations of buffer and dye-free cells. The background light-scattering spectra, of cells without dye, were subtracted from the measured spectra before any comparisons or other analyses were performed. Standardization of dye concentration in the cuvette was done by adding microliter quantities of 5 *N* NaOH to each sample after the spectrum of interest had been taken.

After the adjustment of each sample to a pH between 11 and 12, a calibration spectrum was taken. Since the calibration spectra all had the same shape, the assumption was then made that small height differences between the calibration spectra of various samples were due to small variations in concentration of dye in the cuvette. The spectra of interest were then adjusted accordingly in order to correct for this effect.

Spectral Measurements of pH. For specta without cells or ethanol, 0.99 ml of buffer at the appropriate pH, with or without BSA, was placed in a glass cuvette (1-cm path length) and the pH was checked. Then 5 μg/ml (final concentration) of C.SNARF-1 was added and a spectrum was taken, followed by a calibration spectrum at high pH.

Spectral Measurements in Ethanol/Buffer. For spectra with ethanol, 0.495 ml of buffer and 0.495 ml of 95% EtOH were placed in the cuvette and the pH was checked and adjusted if needed. Then 5 μg/ml (final concentration) of C.SNARF-1 was added and the spectrum and standardization curve were measured.

Spectra of Intracellular C.SNARF-1. For spectra with cells, 0.900 ml of buffer received 0.1 ml of cells (final concentration, 10^6 cells/ml). The pH of the suspension was checked and nigericin (10 μg/ml final concentration) and valinomycin (5 μg/ml final concentration) were added to bring the intracellular pH into agreement with the extracellular value [12]. The sample was allowed to equilibrate for 2 min before spectra were taken. After each spectrum on intact cells, the suspension was sonicated to release the dye and a new spectrum was taken. Following that, the pH was adjusted to pH 11–12 and a standardization spectrum was taken. All spectra were measured at 37°C.

Spectral Analysis. Dye spectra were analyzed on a PC computer with a program [13] which used a least-squares algorithm to find the combination of the acidic-form and basic-form "basis spectra" which best fit the measured curve at each pH. It was assumed that the measured spectrum $S(\lambda)$ could be approximated by a linear combination of basis curves $S_{lo}(\lambda)$ and $S_{hi}(\lambda)$. This has the form

$$S(\lambda) \simeq \alpha S_{lo}(\lambda) + \beta S_{hi}(\lambda) \qquad (1)$$

and the program calculated the best values for α and β. It then constructed mathematically the composite spectrum $\alpha S_{lo}(\lambda) + \beta S_{hi}(\lambda)$ as a check. As long as the original acid-form and the basic-form "basis" spectra were measured with equal concentrations of dye, then α and β represented the relative concentrations of the dye which was in the acidic state and the basic state in the sample being analyzed. The α and β were then related to the pH in the cell and the pK_a of the dye through the Henderson–Hasselbalch equation.

RESULTS

Intracellular Emission Spectra. C.SNARF-1 emission spectra were significantly altered by the intracellular milieu, as can be seen from the family of curves taken at different fixed values of pH_i which is shown in Fig. 1A. For comparison, spectra are also shown for each sample after it was sonicated to disrupt the cells (without change of pH). The almost three-fold enhancement of fluorescence upon cell disruption can be seen by comparing the scales on the *y* axes in Figs. 1A and B. It is clear that the quenching of C.SNARF-1 intact cells is most pronounced when the dye is deprotonated. This effect was is largely reversed by sonicating the cells, although a comparison of the curves in Fig. 1B with the free-dye spectra in Fig. 2A indicates that the reversal upon sonication may have been incomplete. Because the spectra in Fig. 1B indicate that the C.SNARF-1 immediately after cell disruption was pH sensitive, we conclude that the cells did not contain any unhydrolyzed C.SNARF-AM.

Two types of agent were tested on C.SNARF-1 in buffer to see if either could generate a pattern of spectra which would resemble that of the intracellular dye. These results are shown in Fig. 2. The lower right-hand panel shows the effect of BSA, a previously reported [14] quencher of C.SNARF-1. The lower left-hand panel shows

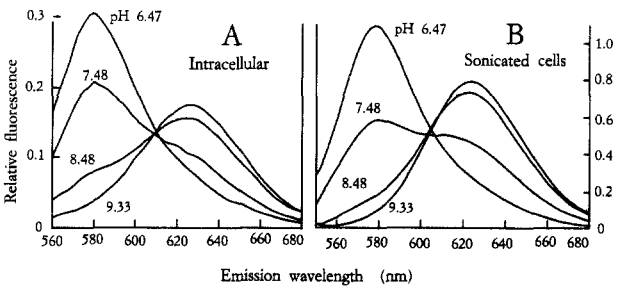

Fig. 1. Emission spectra of C.SNARF-1 within the cell and after liberation by sonication. The buffer was a high-potassium cytosol-like buffer and the intracellular pH was equilibrated with the extracellular by the addition of nigericin and valinomycin [12]. The relative intensities shown on the vertical axes indicate the overall quenching of approximately three-fold which the dye experienced inside the cell. The shape changes between A and B indicates an additional pH-dependent quenching of the deprotonated dye and an increase in the apparent pK_a inside the cell.

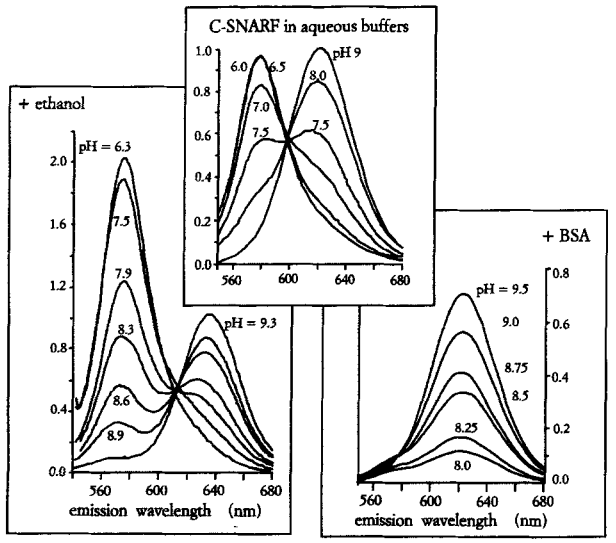

Fig. 2. Emission spectra of C.SNARF-1 in aqueous buffers. The top panel shows pH-dependent spectra in simple buffers. The lower panels show the effects of two classes of compounds which were tested to see if either could mimic the pattern seen for intracellular dye. Protein quenching (10 mg/ml BSA; lower right panel) was completely unlike the intracellular case, whereas ethanol (47%, v/v; lower left panel) produced a result with many qualitative similarities to the intracellular data.

the effect of diluting the buffer 47% with ethanol (in each case keeping pH equal to indicated values). For comparison, the upper panel shows the free dye in simple aqueous buffers. The quenching by BSA exhibited a pattern completely opposite that seen in the cell, since the protonated form of the dye was completely quenched. The effect of ethanol had characteristics much more like the intracellular case in that fluorescence from the protonated form of the dye was relatively stronger and that from the deprotonated relatively weaker.

Calculation of Fully Protonated and Fully Deprotonated "Basis" Spectra. The spectra of the dye in the fully acidic and fully basic states can be calculated from two spectra at known pH using the Henderson–Hasselbalch equation and a value for the effective pK_a. Obtaining the basis spectra in this manner eliminates artifacts that would be associated with experimentally driving the pH to extremely low and high values in order to measure the basis spectra directly. This is particularly useful in the case of the dye which is in intact cells.

The calculation which was used to generate the basis curves starts from two measured spectra, $S_1(\lambda)$ and $S_2(\lambda)$, which were measured on the same dye concentration at known pH values, pH_1 and pH_2, respectively. One then assumes that each of these spectra is mathematically a combination of appropriate amounts of the basis spectra $S_{lo}(\lambda)$ and $S_{hi}(\lambda)$, which correspond to the fully protonated form (low pH limit) and fully deprotonated form (high pH limit). From the definition of pK_a and the Henderson–Hasselbalch equation, one has the following:

$$S_1(\lambda) = \alpha_1 S_{lo}(\lambda) + \beta_1 S_{hi}(\lambda) \quad (2)$$

$$S_2(\lambda) = \alpha_2 S_{lo}(\lambda) + \beta_2 S_{hi}(\lambda) \quad (3)$$

where the amplitudes α and β (proportional to the concentrations of protonated and deprotonated dye, respectively) are given by

$$\alpha_{1,2} = [1 + 10^{(pH_{1,2} - pK_a)}]^{-1} \quad (4)$$

$$\beta_{1,2} = [1 + 10^{(pK_a - pH_{1,2})}]^{-1} \quad (5)$$

At every wavelength λ, the above equations are then solved for the heights of the basis curves $S_{lo}(\lambda)$ and $S_{hi}(\lambda)$. The result is the following expressions, which the computer uses to generate the basis spectra from the measured spectra $S_1(\lambda)$ and $S_2(\lambda)$.

$$S_{lo}(\lambda) = \frac{\beta_2 S_1(\lambda) - \beta_1 S_2(\lambda)}{\alpha_1\beta_2 - \alpha_2\beta_1} \quad (6)$$

$$S_{hi}(\lambda) = \frac{\alpha_1 S_2(\lambda) - \alpha_2 S_1(\lambda)}{\alpha_1\beta_2 - \alpha_2\beta_1} \quad (7)$$

These formulas were applied to the data in Figs. 1 and 2 to obtain the fully protonated and fully deprotonated C.SNARF-1 basis curves in four environments: intracellular, sonicated cells, free dye in aqueous buffer, and free dye in ethanol/aqueous buffer. For display purposes, each pair of basis curves was adjusted in height by a multiplying factor. The factor was the same for both curves in the pair, and it was chosen to make all the fully deprotonated curves have the same height, for ease of comparison. The results are shown in Fig. 3. The method by which the value of pK_a was chosen for the calculation in each of the four cases is described below.

Curve-Fitting Estimates of pK_a for Dye Inside Cells and Out. For each set of spectra measured at known values of pH, two were selected and used, together with an estimated value for pK_a, to generate basis curves. The computer program then used these basis curves to find α and β for the remaining curves of the set. For each of the curves the pH was then calculated from the α and β and the estimated pK_a. That pH was then compared with the measured value. If there was not good agreement, a

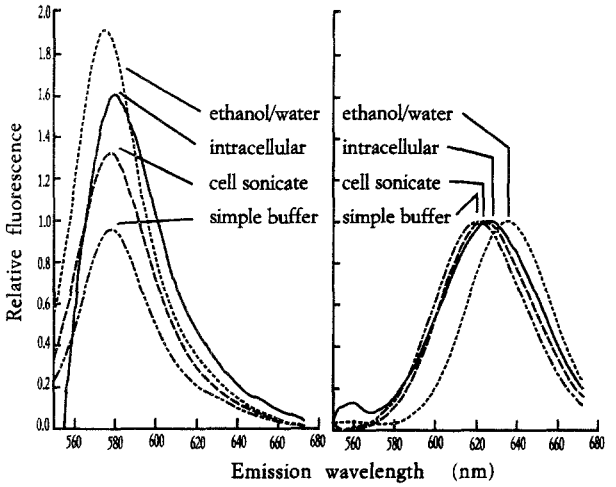

Fig. 3. "Basis" spectra of fully protonated and fully deprotonated C.SNARF-1 in the four environments indicated. Spectra were calculated by the method outlined in the text, which avoided extremes of pH during measurement. Each pair of spectra (each sample) was adjusted in height by an amplitude factor which was chosen to make the deprotonated curve have a height of 1.00, for ease of comparison.

new value of pK_a was adopted, and the process was repeated.

For the free-dye data set as a whole, it was found that if a value of $pK_a = 7.55$ was used to generate the basis curves, then for each of the four curves it was also true that values of pK_a in the range 7.55 ± 0.05 gave agreement between the calculated pH and the known pH at which that curve was measured. In this sense the data showed that the apparent pK_a for C.SNARF-1 in aqueous buffers was 7.55 ± 0.05. A similar procedure was used to determine the pK_a from the measured curves for the sonicated cells (four curves), the free dye in ordinary buffer (seven curves), and the dye in ethanol/aqueous buffer (seven curves).

The results of this calculation were values of 7.55 ± 0.05 for pK_a in both the ordinary buffer and the sonicated cells. The intracellular spectra were well described by a pK_a of 7.75 ± 0.05, and the dye in ethanol/aqueous buffer showed a greater shift, to a pK_a of 8.15.

DISCUSSION

Intracellular Quenching: The intracellular quenching of C.SNARF-1 was clearly pH dependent, since the spectrum in the deprotonated state was affected to a greater extent than in the protonated state. There was also a different extent of wavelength shift in the two states. The interactions with cellular constituents which gave rise to these effects were also responsible for a detectable shift in the apparent pK_a. It went from the value 7.55 ± 0.05, which was seen in ordinary aqueous buffers, to 7.75 ± 0.05. These findings in a cultured cell line were consistent with previous work in splenic lymphocytes [15]. One concludes that spectral and pK_a information obtained from *extra*cellular dye cannot be used as calibrating information for making *intra*cellular dye pH measurements [15]. This and the overall quenching of fluorescence intensity are probably the most significant differences between the ease of use of C.SNARF-1 and that of the pH probe BCECF [15].

The exact interactions which give rise to the intracellular quenching phenomena remain to be determined. However, by comparing quenching of the protonated and deprotonated forms of the dye, along with the apparent pK_a, it was possible to distinguish between two very different types of quenching that were seen in two model cell-free buffer systems. The first was a protein (BSA), which was tested since it had previously been reported to quench C.SNARF-1 [14]. The second was ethanol, which was tested because in earlier work ethanol/aqueous buffers were able to mimic the intracellular spectral shift that was seen in the calcium-chelating dye, Indo-1 [9,16].

The separation of the quenching effects into those on the protonated and those on the deprotonated forms of the dye revealed a dramatic difference between the mechanism of action of BSA and that of ethanol. The former (at 10 mg/ml) quenched the protonated dye, whereas the latter (at 47%, v/v) had a greater quenching effect on the deprotonated dye. These observations, together with the increase in pK_a in ethanol/aqueous buffer, meant that the ethanol–buffer system was much more closely related to the effects observed in the cell than was the protein system.

Data Analysis: The calculation of pH from spectral data requires reference spectral values for fully protonated and fully deprotonated dye. The method used in this work [Eqs. (1)–(6)] obtains this information from spectra at pH values which are known, but less extreme than would be required in order to make direct measurements of fully protonated and deprotonated dye. Avoiding extremes of pH is particularly advantageous in the case of intracellular dye. However, the method does require a value for the effective pK_a of the dye, in order to go mathematically from two measured spectra to the desired "end-point" basis spectra. We dealt with this by assuming a value for pK_a and then testing it by using the resulting basis spectra to analyze other spectra, which were also measured at known pH values but were not used in the calculation of the basis spectra. If the value

of pK_a was the correct one, then the same value which was used to get the basis spectra would also give agreement between the calculated pH and the measured pH for each of the other curve-fitting calculations.

In this manner, if there was a value for pK_a which fit all the data in a set of spectra at known values of pH, one could say the data constituted a measurement of the dye pK_a which yielded that value. The method was applied to the four intracellular dye spectra (pH 6.47 to 9.33) and a value was found for the pK_a, as described. The fact that all the curves were consistent with titration of the dye with a well-defined pK_a was in contrast to the analysis of an earlier experiment [15], in which the basis spectra were not calculated by the extrapolation method of Eqs. (6) and (7). In the analysis of the earlier data, the titration of the intracellular dye showed an apparent failure to approach the deprotonated-dye basis curve fully at high pH.

We attribute the problem with the earlier experiment to the fact that an intracellular spectrum at low pH was used, together with an extracellular spectrum for the high-pH limit. Since the low-pH curve was measured and not extrapolated, it could still have contained a contribution from deprotonated dye. Because the basis curves were required to agree with the measured difference spectrum, this would then have also resulted in extra amplitude for the deprotonated-dye basis spectrum. A result of an amplitude too high for the basis spectrum which was supposed to represent 100% deprotonated dye would have been that, measured against it, actual experimental spectra would have appeared to approach something less than 100% deprotonation as the pH was raised. This is what was seen in that analysis [15].

ACKNOWLEDGMENTS

The authors are indebted to James Whitaker of Molecular Probes, Inc., for extremely helpful discussions and suggestions. This research was supported in part by Grant DCB-9118267 from the National Science Foundation.

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