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Quenching mechanism of quinolinium-type chloride-sensitive fluorescent indicators

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Abstract

Quinolinium based Cl^- sensitive fluorescent indicators have been used extensively to measure intracellular Cl^- activity. To define their fluorescence quenching mechanism, a series of *N*-methyl quinolinium derivatives were synthesized, including *N*-methylquinolinium (Q), 6-methylQ, 6-methoxyQ, 6-chloroQ, 3-bromoQ, 6-aminoQ and *N*-methylisoquinolinium. Stern–Volmer plots for quenching by Cl^-, Br^-, SCN^-, I^-, F^-, OAc^- and CO_3^{2-} from both intensity and lifetime measurements were linear. Bimolecular quenching rate constants (k_q) decreased with increasing anion oxidation potentials and increased with increasing quinolinium reduction potentials. The free energy change for charge transfer (ΔG), calculated from indicator spectral and electrochemical properties, was found to correlate with log k_q. These results suggest that quenching of quinolinium fluorescence in water by anions involves a charge-transfer quenching mechanism. Understanding the mechanism facilitates structure-based predictions of the anion sensitivities of quinolinium indicators to design improved Cl^- indicators with tailored properties. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Fluorescence; Chloride indicators; Anion quenching; Charge transfer

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1. Introduction

Chloride is the major anion in biological systems which plays a role in fluid absorption and secretion, and cell pH and volume regulation. Defective transport of Cl^- across the plasma membrane of epithelial cells is the defect in cystic fibrosis [1]. Cl^- sensitive fluorescent indicators have been used extensively to measure Cl^- activity in living cells and Cl^- transport across cellular and artificial membranes [2–4]. The available Cl^- indicators are heterocyclic compounds with a quarternary nitrogen in which indicator fluorescence is quenched by Cl^- [3,4]. Quinolinium compounds have good sensitivity and selectivity to Cl^-, rapid response to changes in $[Cl^-]$, chemical stability, and low biological toxicity, and are efficiently loaded and trapped in living cells. Structure–activity studies were done to establish an empirical relationship between Cl^- sensitivity and substitution on the quinolinium backbone [5–9]. It was shown that the fluorescence quenching efficiency depended strongly on the position and nature of the substitution on the quinoline ring.

Quenching of the fluorescence of aromatic molecules by anions has been the subject of several investigations. Proposed mechanisms for anion quenching include electron transfer from anions to the excited state fluorophore [10–15], formation of a charge-transfer complex or exciplex formation [16–22], and heavy-atom quenching [23]. Fluorescence quenching of the aromatic heterocyclic compounds acridinium [24], triphenylpyrylium [25], oxonine [26] and acridizinium [23] by substituted aromatic hydrocarbons has been studied in organic solvents. An electron transfer mechanism was proposed based on the correlation between log k_q and ΔG and from the detection of radical pairs by flash photolysis. Lifetime measurements and molecular orbital calculations suggested that the self-quenching of betaine 6-methoxy-(N-3-sulfopropyl) quinolinium in acetonitrile involves an intramolecular electron transfer from the anionic head group to the positively charged chromophore [27]. The only report on the fluorescence quenching of N-aromatic heterocycles in water by anions involved the quenching of 5,6-benzoquinoline and its conjugate acid by Cl^-, Br^-, I^- and SCN^- [28]. The quenching efficiency was found to vary with anion oxidation properties and on the activation energy for the quenching process. Although it was reported that fluorescence quenching was accompanied by an increase in triplet formation, no conclusive quenching mechanism was proposed.

We report here a systematic analysis of the quenching mechanism of a series of synthesized N-methyl substituted quinolinium and isoquinolinium compounds by different anions — Cl^-, Br^-, I^-, F^-, CO_3^-, OAc^- and SCN^-, in aqueous solution.

2. Methods

2.1. Synthesis

All compounds were synthesized by the addition of a 1.5 M excess of iodomethane to the appropriately substituted quinoline and refluxing for 30–60 min without an external solvent. The precipitated solid was washed with acetone several times and then filtered. The indicators were purified by recrystallization from 50% methanol–water and the purity was checked by thin layer chromatography using methanol as solvent. Compound structure was confirmed by NMR. All experiments were done in NaH_2PO_4/Na_2HPO_4 (5 mM) in water (pH 7.2).

2.2. Spectroscopic measurements

Absorption spectra were measured on a Hewlett-Packard photodiode array spectrophotometer (HP 8452A). Fluorescence spectra were measured on a SLM 8000C fluorometer (SLM Instruments, Urbana, IL) equipped with a thermostated cuvette compartment and magnetic stirrer. Fluorescence lifetimes were measured by frequency-domain fluorometry on an SLM 48000MHF fluorometer at 40 modulation frequencies (5–200 MHz). Fluorescence was excited at 322 nm by a He–Cd laser and detected using a Schott glass KV-408 cut-on filter for all compounds except N-methyl-6-aminoquinolinium, where a > 530-nm cut-on filter was used. The

reference fluorophore for lifetime measurements was dimethyl-1,4-bis(4-methyl-5-phenyloxazol-2-yl) benzene (POPOP) in ethanol (lifetime, 1.45 ns) or fluorescein in 0.1 N NaOH (lifetime, 4.0 ns). The singlet excitation energy for the compounds ($E_{0,0}$) was measured from the absorption spectrum.

2.3. Fluorescence quenching measurements

Fluorescence quenching measurements were carried out at peak indicator excitation and emission wavelengths. Microliter aliquots of the sodium salt of the quenching anions (1 M stock) were added to 2 ml of each compound (10 μM, in 5 mM NaH_2PO_4/Na_2HPO_4, pH 7.2). Stern−Volmer constants, K_{sv} (in M^{-1}), were calculated from the slope of a plot of $F_0/F - 1$ vs. quencher concentration $[Q]$ according to the equation, $F_0/F - 1 = K_{sv} [Q]$, where F_0 is compound fluorescence in the absence of quencher and F is the fluorescence in the presence of the quencher.

2.4. Electrochemical measurements

Reduction potentials of the quinolinium compounds were determined by cyclic voltammetry using an EG & G Princeton Applied Research potentiostat/galvanostat (Model 273A). Measurements were carried out in water using 0.1 M KNO_3 as the supporting electrolyte. Standard Ag/AgCl electrode was used as the reference, a glassy carbon electrode as the working electrode, and a platinum mesh as the counter electrode. Cyclic voltammograms were recorded after purging the solution with dry nitrogen for 15 min. The anodic to cathodic peak separations were 120 mV as has been observed for several aromatic heterocycles in water [29]. Anion oxidation potentials were obtained from the literature [21].

2.5. Molecular orbital calculations

Semi-empirical calculations were carried out using the Molecular Orbital Package version 7, using the AM1 Hamiltonian [30]. Geometries for the minimum energy configuration from the PC-MODEL were given as inputs for MOPAC calculation, and no symmetry constraints were imposed during optimization. The optimized geometry corresponding to the minimum value of the heat of formation was obtained for a gradient norm of 0.01. Indicator electron affinities were obtained from the LUMO energies calculated for the optimized geometry. Excitation energies were calculated from the differences in the energy between the HOMO and LUMO.

3. Results and discussion

Fig. 1 shows the fluorescence emission spectrum of *N*-methylquinolinium in water in the presence of indicated concentrations of Cl^-. Except for a decrease in fluorescence, no change in spectral shape and no new long wavelength bands were observed. The fluorescence excitation and absorption spectra of the indicators were similar and not affected by anions (not shown), suggesting that indicator excited state structure/geometry are not modified by the quenching anions.

Fig. 2 shows Stern−Volmer plots for the quenching of *N*-methylquinolinium fluorescence by Cl^- and I^- from steady-state intensities ($F_0/F - 1$) and lifetimes ($\tau_0/\tau - 1$). Stern−Volmer plots
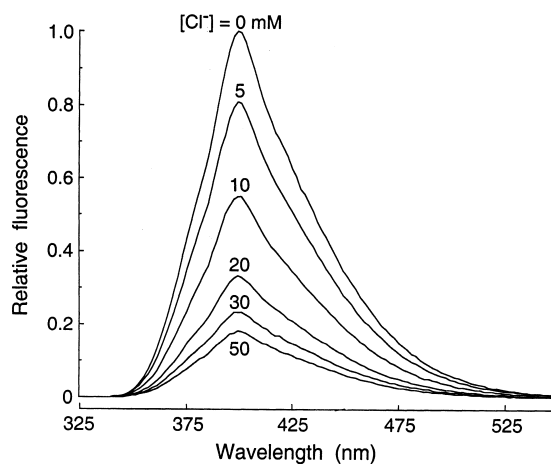

Fig. 1. Emission spectrum of *N*-methylquinolinium iodide (concentration 20 μM) in 5 mM NaH_2PO_4/Na_2HPO_4 in water (pH 7.2) in the presence of specified concentrations of NaCl.
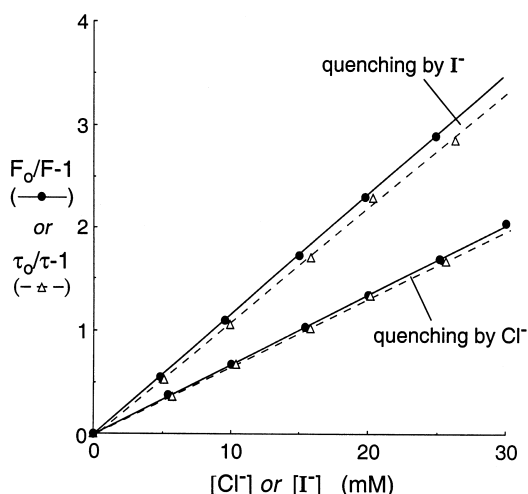
Fig. 2. Stern–Volmer plots for the quenching of N-methyl-quinolinium iodide (20 μM) in 5 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 7.2) by Cl$^-$ and I$^-$ from steady-state intensity and lifetime measurements.

obtained both from steady-state and lifetime measurements were linear for all compounds (correlation coefficients $\geq$ 0.99). The decrease in fluorescence intensity paralleled the decrease in indicator lifetime, confirming a pure dynamic quenching mechanism.

For collisional quenching, the bimolecular quenching rate constant k_q (in M^{-1} s^{-1}) is related to the Stern–Volmer constant (K_{sv}) by the equation: $K_{sv} = k_q \tau_0$, where τ_0 is indicator fluorescence lifetime in the absence of quencher. k_q

values for different indicator–anion combinations are summarized in Table 1 and were in the range 10^8–10^{10} M^{-1} s^{-1}. The k_q value for strong quenchers like I$^-$ is close to the diffusion-limited rate constant (k_{diff}), whereas k_q is 2–3 orders of magnitude less for other anions. The fluorescence quenching of 6-amino-N-methylquinolinium is anomalous, probably because of an intramolecular charge transfer phenomenon, as has been observed in other amino substituted compounds [19,22]. Comparison of k_q for different anions suggests that the quenching efficiency is related to the ease of anion oxidation. Also, quinolinium indicator substitution with an electron withdrawing group increases quenching. Based on these observations, k_q were correlated with the electrochemical properties of the indicators and anions. The measured reduction potentials (E_{red}) of the indicators are listed in Table 1 along with the measured singlet excitation energies $E_{0,0}$.

Fig. 3a shows a close correlation between log k_q for the quenching of N-methylquinolinium fluorescence by different anions. The quenching efficiency decreases with increasing anion oxidation potential. Fig. 3b shows the correlation between log k_q and indicator reduction potential in the excited state ($E^*_{red} = E_{red} + E_{0,0}$) for the quenching of a series of quinolinium indicators by Cl$^-$. k_q increases with increasing indicator reduction potential. The dependence of k_q on the redox properties of the quinolinium indicators

Table 1
Spectroscopic, quenching and electrochemical properties of quinolinium indicators[a]

	Compound	Cl$^-$	Br$^-$	I$^-$	SCN$^-$	CO$_3^{2-}$	OAc$^-$	F$^-$	E_{red} (V)	$E_{0,0}$ (eV)	τ_0 (ns)
I	N-Methylquinolinium	1.14	1.50	1.83	1.29	0.58	0.43	0.02	−0.86	3.90	14.5
II	N-Methlisoquinolinium	0.42	0.62	0.85	0.66	0.20	0.10	0.03	−1.04	3.90	15.2
III	N-Methyl-6-methyl quinolinium	0.55	0.85	1.18	0.93	0.21	0.25	0.03	−0.96	3.83	19.5
IV	N-Methyl-6-methoxy quinolinium	0.44	0.85	1.18	0.91	0.12	0.04	0.01	−1.06	3.24	25.5
V	N-Methyl-6-chloro quinolinium	0.46	1.09	3.20	0.80	0.40	0.13	0.03	−0.70	3.86	1.0
VI	N-Methyl-3-bromo quinolinium	1.47	1.73	4.50	1.97	0.96	0.77	0.10	−0.54	3.90	3.0
VII	N-Methyl-6-amino quinolinium	0.02	0.13	1.26	1.17	1.10	0.33	0.11	−0.45	2.71	3.8

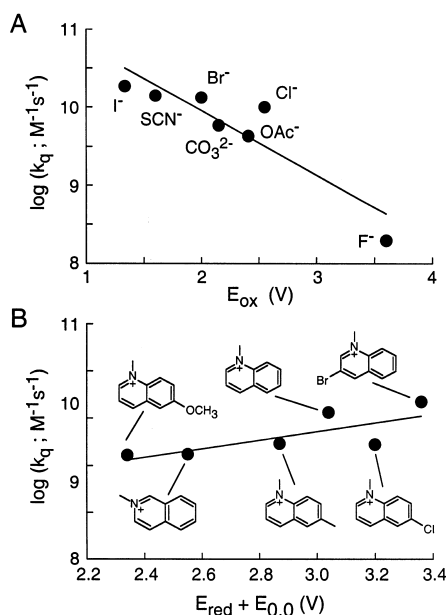[a] Units in k_q (10^{10} M^{-1} s^{-1}) except where stated otherwise.

Fig. 3. Semi-logarithmic plot of the bimolecular quenching rate constant k_q, for quenching of N-methylquinolinium iodide fluorescence by indicated anions vs. anion oxidation potentials. (B) Semi-logarithmic plot of k_q for fluorescence quenching of the indicated compounds by Cl^- vs. indicator reduction potentials in the excited state ($E_{red} + E_{0,0}$).

and the anions suggest a quenching mechanism involving charge transfer from the anion to the indicator.

The charge-transfer process in solution consists of fluorophore and quencher diffusion to form an encounter complex, intermolecular charge transfer, followed by the quenching and dissociation of the complex. The free energy change (ΔG) for the formation of the charge-transfer complex (exciplex) is given by the Rehm–Weller expression [31],

$$\Delta G = E_{ox} - E_{red} - E_{0,0} + C \qquad (1)$$

where, E_{ox} is the oxidation potential of the donor, E_{red} is the reduction potential of the acceptor, $E_{0,0}$ is the singlet excitation energy of the fluorophore, and C is the coulombic energy released upon charge transfer [given as $-e_0^2/4\pi\varepsilon_0\varepsilon a$, where ε_0 is the permitivity of free space, ε is the solvent dielectric constant, and a is the encounter distance between the electron donor and acceptor

taken as 6–9 Å]. C was calculated to be 0.02–0.03 eV, representing a minor contribution to ΔG in Eq. (1). k_q is related to ΔG by $\Delta G = -RT\ln k_q$. Fig. 4 shows a plot of log k_q vs. ΔG. As ΔG becomes more negative quenching increases; for highly negative ΔG, k_q approaches k_{diff}. A linear correlation was obtained between log k_q and ΔG,

$$\log k_q = 9.19 - 0.73\Delta G \qquad (2)$$

This linear correlation supports a quenching mechanism involving excited state charge transfer from the anion to the quinolinium indicator.

The excited state charge transfer complex that is formed during the quenching of quinolinium indicators by anions is probably de-excited to the ground state by a non-radiative pathway, since no exciplex emission is observed in the presence of the quenchers. Some of the anions used in our study are heavy atoms and it was observed by Carrigan et al. [28] that quinolinium fluorophore triplet formation is enhanced in the presence of quenching anions like iodide. Thus the de-excitation pathway for the charge complex for some anions may be enhanced intersystem crossing. Furthermore, the quenching cannot be explained by pure electron transfer phenomenon (Rehm–Weller theories [31], Marcus [32] theories) since
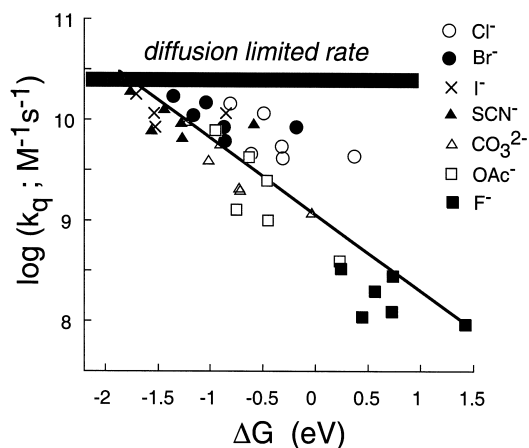

Fig. 4. Fluorescence quenching rate constants k_q as a function of free energy change ΔG for electron transfer for indicated anions. Bar shows predicted diffusion-limited rate constant.

k_q is less than k_{diff} at exothermic ΔG values and the correlation between log k_q and ΔG is imperfect. It is thus likely that parallel quenching processes are present, including charge transfer, electron transfer and heavy atom quenching.

For highly efficient quenchers like iodide, the k_q values are of the order of k_{diff}. For comparison purpose, we estimated the diffusion-limited rate constants using the Debye expression [33],

$$k_1 = 4\pi N z_A z_B e^2 (D_A + D_B) N 1000$$
$$/4\pi\varepsilon_0\varepsilon RT \left[\exp(z_A z_B e^2/4\pi\varepsilon RTa) - 1\right] \tag{3}$$

where z_A, z_B are the charges of the anion and the indicator ion, respectively, and D_A and D_B are the corresponding diffusion coefficients. Using indicator diffusion coefficients (estimated from the Stokes−Einstein equation and Van der Waals radii) of $(3−4)\times 10^{−6}$ cm^2 s$^{−1}$ and anion diffusion coefficients from the literature [11,28], the diffusion-limited rate constants were in the range of $(1.9−2.2)\times 10^{10}$ M$^{−1}$ s$^{−1}$ (Fig. 4). The measured k_q values were nearly 1−2 orders of magnitude lower than the diffusion-limited rate for quenching by Cl$^−$, F$^−$, CO$_3^{2−}$, OAc$^−$, 2−4 times lower for quenching by Br$^−$ and SCN$^−$, and comparable in case of I$^−$.

Further evidence in support of a charge transfer quenching mechanism was obtained by examination of solvent effect. Solvent can affect the efficiency of quenching by changes in polarity and/or viscosity. Measurements were carried out in solutions made viscous by adding different weight percent sucrose (Fig. 5a) to study viscosity effects. To study polarity effect, quenching experiments were done in water/acetonitrile mixtures (Fig. 5b) in which both solvent polarity and viscosity decreases as the mole fraction of acetonitrile increases [34]. N-Ethyl-6-methoxyquinolinium iodide and N-(3-sulfopropyl)-6-methoxyquinolinium are the representative indicators chosen for this study because of their improved solubility in acetonitrile/water mixtures. The quenching constant decreased by 1.6-fold for a twofold increase in viscosity (Fig. 5a) whereas
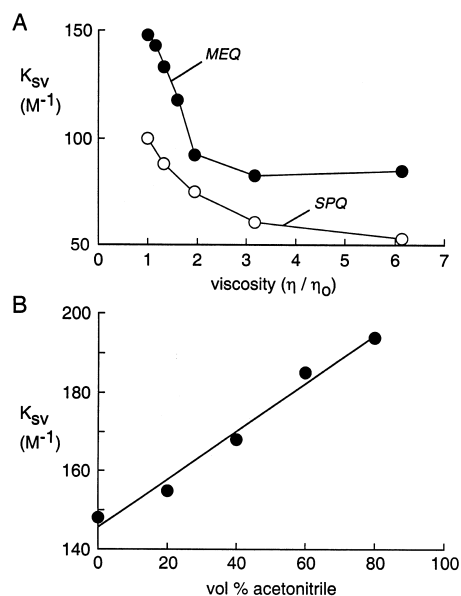

Fig. 5. Effect of solvent viscosity and polarity on quenching. (A) Stern−Volmer constants for the quenching of 6-methoxy-N-ethylquinolinium iodide (MEQ) and 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) fluorescence by Cl$^−$ as a function of solution viscosity. Solution viscosity was varied by the addition of sucrose. (B) Stern−Volmer constants for quenching of 6-methoxy-N-ethylquinolinium iodide fluorescence quenching by Cl$^−$ in acetonitrile/water mixtures.

there was only a 1.3-fold increase in quenching constant for a twofold decrease in viscosity produced by adding acetonitrile (Fig. 5b). The lesser increase in K_{sv} in acetonitrile/water mixture is due to the decrease in polarity (dielectic constant for water = 80: for acetonitrile = 36). These data support the involvement of a charge transfer mechanism for quenching of quinolinium fluorescence by anions.

Together our study provides evidence that the quenching of quinolinium indicators by anions is primarily charge transfer-mediated. An understanding of the quenching mechanism suggests the possibility of structure-based computational prediction of k_q for de novo design of Cl$^−$ indicators with improved properties. To predict k_q theoretically, ΔG is computed from Eq. (1). Since anion E_{ox} are available in the literature, it is necessary to compute only indicator E_{red} and $E_{0,0}$. Semi-empirical computations of these
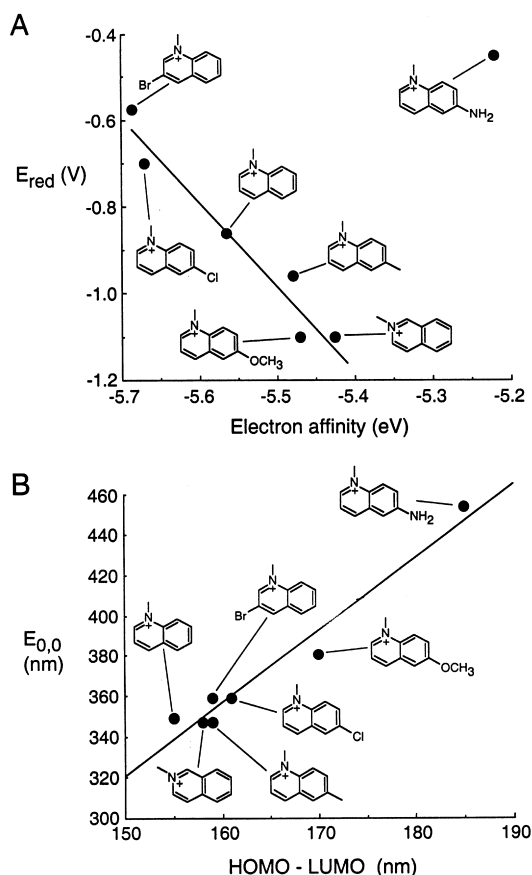
Fig. 6. Structure-based prediction of indicator anion sensitivity. (A) Measured indicator E_{red} vs. theoretically computed electron affinity (E.A.) (see text for explanations). (B) Measured indicator $E_{0,0}$ vs. theoretically computed $E_{HOMO-LUMO}$.

parameters were carried out for the quinolinium indicators as described in Section 2. Fig. 6a shows the relationship between computed electron affinities (E.A.) and experimentally determined E_{red} ($E_{red} = -11.9 - 1.99$ E.A.) and Fig. 6b shows the correlation between $E_{HOMO-LUMO}$ and experimentally measured $E_{0,0}$ ($E_{0,0} = 3.68$ $E_{HOMO-LUMO} - 231$). (The electron affinity of 6-amino-N-methyl quinolinium did not fit the prediction, probably because of hydrogen bonding effects).

As a test of the computation approach, k_q values were predicted for two quinolinium compounds that were not included in the analysis, but for which k_q are available from the literature.

Computed ΔG values were -0.28 eV for N-methyl-2-methylquinolinium and -0.04 eV for N-methyl-7-methylquinolinium. Corresponding k_q (obtained from Eq. (2)) were 2.46×10^9 M^{-1} s^{-1} for N-methyl-2-methylquinolinium and 1.64×10^9 M^{-1} s^{-1} for N-methyl-7-methylquinolinium, in reasonable agreement with experimentally reported values of 4.5×10^9 M^{-1} s^{-1} and 1.06×10^9 M^{-1} s^{-1}, respectively [5].

In summary, the results here indicate the quenching of quinolinium indicators by anions is dynamic and mediated through a non-emissive excited state charge transfer complex. The correlation between quenching efficiencies and indicator/quencher redox properties provides a rational explanation for empirical structure−activities studies reported over the past decade. Understanding the mechanism may facilitate structure-based predictions of the anion sensitivities of quinolinium indicators to design improved Cl^- indicators with tailored properties.

As the mechanism of quenching of the quinolinium type indicators involves a dynamic excited state charge transfer process, the in vivo Cl^- sensitivity and brightness of these indicators is limited because of the parallel quenching by different intracellular quenchers like proteins. The brightness and intracellular sensitivity can be improved by designing a different class of indicators that will bind anions specifically in the ground state. It was reported recently [35] that a mutant form of green fluorescent protein (GFP) is sensitive to anions by a static quenching mechanism. GFP-based indicators could be used for intracellular Cl^- measurements with excellent sensitivity.

Acknowledgements

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