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Fluorescence Quenching of Anthracene in Charged Micelles by Pyridinium and Iodide Ions†

Henry J. Pownall and Louis C. Smith*,‡

ABSTRACT: The accessibility of fluorescent groups in proteins can be determined with charged quenching agents which provide information about conformational topography and local charge distribution surrounding the fluorescent moiety. Micelles of sodium dodecyl sulfate and cetyltrimethylammonium bromide containing anthracene were used to characterize the quenching properties of pyridinium and iodide ions in a model system where the fluorophore and charge distribution are known with certainty. Iodide ions were effective quenchers only with positively charged cetyltrimethylammonium bromide micelles. Pyridinium ions quenched anthracene fluorescence only in negatively charged sodium dodecyl sulfate micelles. The quenching constants for pyridinium chloride in ethanol and sodium dodecyl sulfate micelles were 42 and 520 M^{-1}, respectively. For potassium iodide the corresponding values in ethanol and cetyltrimethylammonium bromide micelles were 17 and 418 M^{-1}. When cetylpyridinium ions were incorporated into the cetyltrimethylammonium bromide micellar structure the quenching constant was 348 M^{-1}. If the micelle–anthracene systems interacted with an ion of opposite charge, the quenching constant, K_{sv}, was about ten times greater than that predicted by diffusion controlled kinetics. If the quencher and the micelle–anthracene system had like charges, K_{sv} was practically nil. These results establish that iodide and pyridinium ions exhibit vastly different quenching capacities which are dependent on local charge distribution. Adequate interpretation of quenching studies with biological systems such as proteins, lipoproteins, and membranes where the microscopic environment is unknown can be more adequately interpreted if both probes are employed to obtain the necessary complementary information.

The topography and conformation of proteins in solution have been investigated with probes which perturb the spectral properties of aromatic amino acids. Fluorescence quenching constants derived from iodide ion interaction with lysozyme have been used to estimate the exposure of tryptophan residues to the solvent (Lehrer, 1967, 1971). For the native protein about one-half the tryptophan residues were accessible at pH 5.3, whereas all of the tryptophan fluorescence was quenched after unfolding in 6 M guanidinium chloride. The presence of charged groups near tryptophanyl side chains was inferred by the dependence of the quenching on pH. Intramolecular complexes formed between *N*-methylnicotinamide chloride and on the solvent-available tryptophan in lysozyme and α-lactalbumin also provided information about the accessibility of the aromatic amino acid residue (Deranleau *et al.*, 1969; Bradshaw and Deranleau, 1970; Robbins and Holmes, 1972). The more available residue gave a strong charge-transfer band, comparable to that obtained with the free amino acid in solution (Deranleau and Schwyzer, 1970). Weak or absent charge-transfer absorption bands indicated no interaction between the acceptor and the residue buried in the proteins.

In solutions where both fluorophore and quencher move freely, quenching of fluorescence occurs through a collisional mechanism described by the following processes (Parker, 1968; Birks, 1970a).[1]

	Process	Rate
$F + h\nu \longrightarrow F^*$	Absorption	I_a
$F^* \longrightarrow F + \Delta$	Sum of nonfluorescent events deactivating the lowest singlet state	$k_{r1}[F^*]$
$F^* \longrightarrow F + h\nu'$	Fluorescence	$k_f[F^*]$
$F^* + Q \longrightarrow F + Q + \Delta$	Singlet quenching	$k_q[F^*][Q]$

The quenching agent is Q; the ground state and excited state of the fluorophore are represented by F and F*, respectively. Steady-state treatment of these equations gives

$$\varphi_f^0/\varphi_f = 1 + k_q[Q](k_f + k_{r1})^{-1} = 1 + K_{sv}[Q] \quad (1)$$

or

$$(\varphi_f^0/\varphi_f) - 1 = k_q\tau_0[Q] = K_{sv}[Q] \quad (2)$$

where the fluorescence decay time without quencher is

$$\tau_0 = 1/(k_r + k_{r1})$$

Equation 2 is the familiar Stern–Volmer equation for fluorescence quenching (Stern and Volmer, 1919). Since relative quantum yields are proportional to relative intensity, the Stern–Volmer quenching constant, K_{sv}, is obtained from a plot of $(I_0/I) - 1$ *vs.* [Q], where I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively.

In solutions where both fluorophore and quencher can move freely the value of k_q approximates that of a diffusion controlled process. The accessibility of the fluorophore to the quencher will be reflected in the measured value of k_q and of K_{sv} obtained from a plot described by eq 2. The effectiveness of these charged probes as quenching agents should be dependent on the structure and local charge distribution in

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[1] Other processes deactivating the triplet state have been omitted.

TABLE I: Quenching Constants for Anthracene and Pyrene in Ethanol and Detergent Micelles.

Fluorophore	Quencher	Solvent[b]	K_{sv}
Anthracene	Pyridinium chloride	Ethanol	42 ± 1
Anthracene	Cetylpyridinium chloride	Ethanol	45 ± 0.5
Anthracene	Potassium iodide	Ethanol	17 ± 0.5
Anthracene	Pyridinium chloride	SDS micelle	520 ± 20
Anthracene	Potassium iodide	CTAB micelle	418 ± 15
Anthracene	Cetylpyridinium chloride	CTAB micelle	348 ± 12
Anthracene	Potassium iodide	SDS micelle	<1.0
Anthracene	Pyridinium chloride	CTAB micelle	<1.0
Anthracene	Quinolinium chloride	SDS micelle	1575 ± 50
Pyrene	Pyridinium chloride	Ethanol	175 ± 15[a]
Pyrene	Pyridinium chloride	SDS micelle	2600 ± 40[a]

[a] These values represent a lower limit because the fluorescence lifetime is sensitive to oxygen. [b] SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide.

the immediate environment of the aromatic residues in the proteins. In this study we have characterized the quenching properties of two probes, iodide and pyridinium ions, in charged micelles of sodium dodecyl sulfate and cetyltrimethylammonium bromine containing anthracene as the fluorophore. Subsequent studies with lipoproteins are described in the following papers (Pownall and Smith, 1974; Jackson *et al.*, 1974).

Materials and Methods

Potassium iodide (Baker Analyzed reagent), sodium iodide (Baker Analyzed reagent), anthracene (Aldrich, puriss), and ethanol (U.S.I., absolute) were used as received from the manufacturer. Cetylpyridinium chloride (Matheson Coleman and Bell) and cetyltrimethylammonium bromide (Eastman) were recrystallized from ethanol–ether. Sodium dodecyl sulfate (Fisher Scientific Co.) was recrystallized from ethanol. Pyridinium chloride, quinolinium chloride, and acridinium chloride were prepared by bubbling hydrogen chloride through an ether solution of the parent base (Fieser and Fieser, 1967). The resulting precipitate was recrystallized from ethanol–ether and dried *in vacuo* over P_2O_5 before use.

The emission spectra were recorded on an Aminco-Bowman spectrofluorimeter at ambient temperatures. The spectra of 2 ml of ethanol or 1.0 M detergent containing 10^{-5} M anthracene in a 1-cm^2 quartz fluorescence cell were obtained initially. The quenching agent, in 10-μl aliquots of 1.0 M pyridinium chloride or 5.0 M KI, was added to the solution with a 100-μl syringe. After each addition, the solution was thoroughly mixed and the emission spectrum recorded. Corrections for dilution were found to be unnecessary because the total volumes of quenching solution added were 2% or less, except for the experiments where the micelle and quencher had the same charge. In these experiments (Table I), the fluorescence intensity was attenuated to the same degree, within experimental error, when volumes as large as 1 ml of either solvent or quenching solutions were added. To prevent I_3^- formation, 10^{-4} M $Na_2S_2O_3$ was included in the 5.0 M KI solutions.

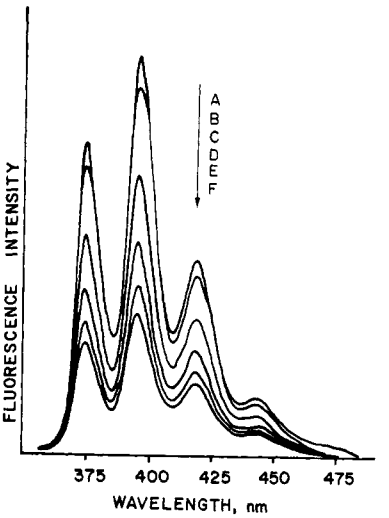

FIGURE 1: Effect of pyridinium ion on anthracene fluorescence spectrum. Anthracene concentration in ethanol was 10 μM. Pyridinium chloride concentrations for spectra A–F were 0, 2.5, 12.5, 25, 37.5, and 50 mM, respectively. Excitation and analyzing slit widths were 2.0 and 0.5 nm, respectively. The excitation wavelength was 365 nm, employing band passes of 5 nm on both excitation and analyzing monochromators.

The labeled detergents were prepared by sonication of a 1.0 M solution of detergent with anthracene and filtration through a 0.45-μ Millipore filter to remove undissolved anthracene. The filtrate was diluted with 1.0 M detergent until the anthracene absorbance at 375 nm was between 0.1 and 0.5.

Results

The effect of pyridinium chloride on the fluorescence spectrum of anthracene in ethanol is shown in Figure 1. With increasing amounts of the quencher, the fluorescence efficiency was diminished as predicted by Stern–Volmer kinetics. This is confirmed by the plot of the function, $(I_0/I) - 1$, as a linear function of pyridinium chloride concentration shown as curve A in Figure 2. The slope of this line corresponds to the quenching constant, K_{sv}. The value obtained for quenching of anthracene fluorescence by pyridinium ion was 42 ± 1 M^{-1}. The specific rate constant, k_q, for this interaction was obtained from $K_{sv} = k_q'\tau$, where the fluorescence lifetime, τ, was 4.9×10^{-9} sec (Berlman, 1971). The value for k_q, 8.6×10^9 M^{-1} sec^{-1}, was comparable to the diffusion controlled rate constant, k_d, which was 5.4×10^9 M^{-1} sec^{-1}. This rate constant for the bimolecular process in ethanol was evaluated from (Bowen, 1954)

$$k_d = 8RT/3000\eta$$

where R is the gas constant, T is the temperature, and η is the viscosity of ethanol, which is 1.078 cP at 25° (Riddick and Bunger, 1970). The similar values of k_d and k_q suggest that quenching of anthracene fluorescence by pyridinium ion in ethanol is a diffusion controlled process.

The efficiencies with which the charged probes, iodide and pyridinium ions, quench anthracene fluorescence in micelles of identical and opposite charges were studied in a variety of anionic and cationic detergents. Solubilization of anthracene was monitored by both fluorescence and absorption spectra in micelles of phenyltrimethylammonium bromide, phenyltriethylammonium bromide, sodium dodecyl sulfate, cetyltrimethylammonium bromide, and cetylpyridinium bromide. In each surfactant, the anthracene absorption spectrum

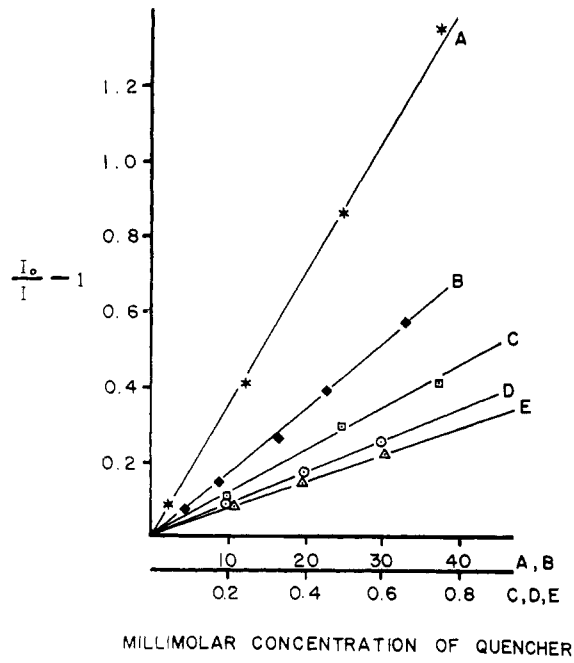

FIGURE 2: Stern–Volmer plots of anthracene quenching. Plots A and B were obtained in ethanol with pyridinium chloride and potassium iodide, respectively. Plot C is the effect of pyridinium chloride in sodium dodecyl sulfate micelles. Potassium iodide and cetylpyridinium chloride effects in cetyltrimethylammonium bromide micelles are represented by plots D and E, respectively.

was shifted to longer wavelengths relative to its spectrum in hydrocarbons. The emission spectrum of anthracene in these micelles also appeared at longer wavelengths than were observed in a hydrocarbon solvent. This wavelength shift is a consequence of the more polar environment of the micellar interior (Becker, 1969). The long-chain alkyl groups were, as expected, most efficient in solubilizing anthracene (Elworthy *et al.*, 1968). The anthracene fluorescence spectrum was observed in all these detergents except cetylpyridinium chloride in which no fluorescence could be detected. The interaction of pyridinium and iodide ions with anthracene in ethanol and in charged micelles are depicted as Stern–Volmer plots in Figure 2. Iodide ion has been employed previously as a quenching agent (Lehrer, 1971) and was included in this study to demonstrate that these two quenching probes should be used in conjunction with each other. The effects of charge in ethanol are minimized. Reference data were obtained in this solvent. The quenching constants for the interaction of pyridinium ion with anthracene in sodium dodecyl sulfate micelles were 420 M^{-1} and 520 M^{-1}, for iodide ion with cetyltrimethylammonium bromide micelles labeled with anthracene. These results are summarized in Table I. In systems consisting of sodium dodecyl sulfate–anthracene plus iodide and cetyltrimethylammonium bromide–anthracene plus pyridinium ion, K_{sv} was less than 1.0. Quinolinium ion quenched anthracene fluorescence effectively in sodium dodecyl sulfate micelles whereas pyridine and triphenylammonium chloride were comparatively ineffective. Acridinium chloride also quenched effectively but the fluorescence of the acridinium ion precluded an accurate determination of K_{sv}. Stoichiometric addition of sodium hydroxide to pyridinium quenched systems restored the fluorescence intensity. An active quenching agent was generated by the protonation of a nitrogen heterocycle but niether the parent base nor ordinary amines possessed the high quenching property of the quaternized azaaromatics. The quenching constant of cetylpyridinium chloride in cetyltrimethylammonium bromide micelles was the same order of magnitude as K_{sv} for iodide ion in cetyltrimethylammonium bromide micelles.

Discussion

Micelles of long-chain detergents and anthracene were selected as model systems to illustrate unequivocally the dependence of quenching on charge–charge interaction. The relatively high fluorescence quantum yield, $\varphi_f = 0.36$, of anthracene (Berlman, 1971) permits accurate measurement of low quantities of fluorophore. The short fluorescence decay time, 4.9 nsec, makes the fluorescence quantum yield insensitive to small changes in oxygen concentration. Anthracene is insoluble in water and therefore confined to the hydrocarbon interior of the micelle. Additionally, solubility of anthracene in ethanol provided a homogeneous system for reference to examine quenching characteristics of iodide and pyridinium ion in the absence of a charged region surrounding the fluorophore.

Neither iodide ion nor pyridinium ion has appreciable absorption above 275 nm, thereby excluding possible artifacts from excitation. It has been shown that iodide ion forms charge-transfer complexes with tryptophan and that pyridinium ion forms charge-transfer complexes with nitrogen containing heterocyclic compounds and indole derivatives (Briegleb *et al.*, 1969). We have observed a 25% decreased absorption of anthracene, 10^{-5} M, in ethanolic pyridinium chloride, 2×10^{-2} M, which might have arisen from charge-transfer interaction.

The negatively charged iodide ion was only an effective quencher of micelle systems in which the positively charged cetyltrimethylammonium bromide system was employed. Conversely, pyridinium ion was effective in quenching anthracene fluorescence in negatively charged sodium dodecyl sulfate micelles but ineffective with cetyltrimethylammonium bromide micelles.

The absence of detectable interaction between quenching agents and micelles of identical charges is expected because of electrostatic repulsion. When cetylpyridinium chloride was employed as the quencher as part of the micelle structure with cetyltrimethylammonium bromide, hydrophobic interactions were stronger than the repulsive electrostatic forces. Interaction of the micelle–anthracene system with a quenching agent of opposite charge yields quenching constants, K_{sv}, about ten times greater than that predicted by diffusion controlled kinetics and would result from electrostatic attraction.

The large K_{sv} value obtained by pyridinium ion quenching of pyrene in sodium dodecyl sulfate micelles and in ethanol is consistent with the much longer fluorescence lifetime, τ = 500 and 4.9 nsec for pyrene and anthracene, respectively (Birks, 1970b).

The mechanism for quenching could be static or a dynamic process (Vavilov, 1929; Vaughan and Weber, 1970; Weber, 1948). The former involves interaction of a quenching sphere with the fluorophore, whereas the latter mechanism requires that the quencher and fluorophore collide. This mechanism is not operative in rigid systems. Whether or not the mechanism of fluorescence quenching involves a collision process or formation of a nonluminescent exiplex between the tryptophan and the quencher, Stern–Volmer plots will still be linear. In the former case, the slope of this plot, K_{sv}, will be the product of the fluorescence lifetime of the fluorophore and the bimolecular rate constant for the reaction. In the latter instance the K_{sv} value will actually be the equilibrium con-

stant for the exiplex formation (Carrión *et al.*, 1968; Parker, 1968). These two processes are not easily distinguished. In either case, however, close proximity of fluorophore and quencher is prerequisite for fluorescence quenching so that this mechanistic ambiguity does not interfere with quenching studies designed to determine the exposure of fluorophore to the solvent.

We believe that these results are important in studies of charged ions as quenching probes of protein, membrane, and lipoprotein structure. In this study iodide and pyridinium ions exhibited vastly different quenching capacities which were dependent upon the micelle and quencher charges. Maximum quenching efficiency was observed with species with opposite charges. The interpretation of results obtained on relatively unknown systems with only one probe is potentially ambiguous. For example, if the microenvironment of the fluorophore were positively charged, the rate of quenching by iodide ion would be enhanced. Conclusions about the accessibility of tryptophan residues might be erroneous. Both probes provide complementary information. The utility of iodide ion and pyridinium ion as quenching agents of tryptophan fluorescence is illustrated by studies of lipoproteins in the following papers (Pownall and Smith, 1974; Jackson *et al.*, 1974). Changes in the accessibility of tryptophan residues and in the average charge distribution of the microenvironment of the fluorophore occurred as the result of phospholipid binding to plasma apolipoproteins.

Acknowledgments

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