J|A|C|S
ARTICLES
Published on Web 09/19/2002

Electrochromic Modulation of Excited-State Intramolecular Proton Transfer: The New Principle in Design of Fluorescence Sensors

Andrey S. Klymchenko*,†,‡,§ and Alexander P. Demchenko*,†,‡

Contribution from the TUBITAK Research Institute for Genetic Engineering and Biotechnology, 41470, Gebze-Kocaeli, Turkey, Department of Chemistry, Kyiv National Taras Shevchenko University, 01033 Kyiv, Ukraine, and A. V. Palladin Institute of Biochemistry, 9 Leontovicha Street, 01030 Kyiv, Ukraine

Received July 12, 2002

Abstract: Internal Stark effect (or internal electrochromy) consists of the shift of light absorption and emission bands under the influence of electric field produced by proximal charges. In the studies of 3-hydroxyflavone (3HF) derivatives exhibiting the excited-state intramolecular proton transfer (ESIPT), we describe a new phenomenon − a very strong internal electrochromic modulation of this reaction. Fluorescence spectra of 3HF derivatives with charged groups attached to the chromophore from the opposite sides without π-electronic conjugation, *N*-[(4′-diethylamino)-3-hydroxy-6-flavonyl]methyl-*N*,*N*-dimethyloctylammonium bromide and 4-{4-[4′-(3-hydroxyflavonyl)]piperazino}-1-(3-sulfopropyl)pyridinium, were compared with those of their neutral analogues in a series of representative solvents. The introduction of the proximal charge results in shifts of absorption spectrum and of both normal (N*) and tautomer (T*) emission bands, which correspond to initial and phototautomer states of the ESIPT reaction. The observed shifts are in accordance with the Stark effect theory. The direction of the shift depends on the position of the proximal charge with respect to the chromophore. The magnitude of the shift depends strongly on the solvent dielectric constant and on screening or unscreening produced by addition of the hydrophobic salts. In all of these cases, the spectral shifts are accompanied by extremely strong variations of relative intensities of N* and T* emission bands. This signifies a strong influence of internal electric field on the ESIPT reaction, which produces a dramatic change of emission color. Thus, the coupling of the initial electrochromic sensory signal with the ESIPT reaction allows for the breaking of the limit in magnitude of response inherent to common electrochromic dyes. This suggests a new principle of designing the ultrasensitive electrochromic two-wavelength fluorescence sensors and probes for analytical chemistry, macromolecular science, and cellular biology.

Introduction

The most desirable property of a molecular probe or sensor based on fluorescence is the ability to respond to applied perturbation in a highly selective and sensitive manner by dramatic change of emission color. For this purpose, organic photochemistry can offer many organic dye systems,[1] but only the systems applying the intramolecular transfer of electronic charge have found so far intensive practical application.[2] The fast responding probes for biomembrane potential belong to this category. Their electrochromic response is based on direct interaction of the ground- and excited-state dipoles of the chromophore with the applied electric field.[3] Despite the successful application of electrochromic probes in cellular research,[4] it is limited by their relatively low sensitivity. Thus, the best charge-transfer electrochromic dyes show only about 10% ratiometric response per 100 mV change in transmembrane electric potential, and this is believed to be already close to the physical limit.[3] Is there any possibility of breaking this limit by coupling the electrochromism with an excited-state reaction?

Very promising in this respect are the dyes of the 3-hydroxyflavone (3HF) family.[5] They exhibit the excited-state intramolecular proton transfer (ESIPT) reaction between the normal excited state (N*) and phototautomer state (T*).[6] Both states

* To whom correspondence should be addressed. Tel.: +90 262 641 2300. Fax: +90 262 641 2309. E-mail: andrey@rigeb.gov.tr.
† TUBITAK.
‡ Kyiv National Taras Shevchenko University.
§ A. V. Palladin Institute of Biochemistry.

(1) De Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515−1566.
(2) (a) Valeur, B. In *Molecular Luminescence Spectroscopy. Methods and Applications. Pt 3*; Schulman, S. G., Ed.; Wiley-Interscience: New York, 1993; p 25. (b) Valeur, B. *Molecular Fluorescence*; Wiley VCH: Weinheim, 2002.
(3) (a) Cross, D.; Loew, L. M. *Methods Cell Biol*. **1989**, *30*, 193−218. (b) Loew, L. M. *Methods Cell Biol*. **1993**, *38*, 194−209.
(4) (a) Loew, L. M.; Scully, S.; Simpson, L.; Waggoner, A. S. *Nature* **1979**, *281*, 497−499. (b) Montana, V.; Farkas, D. L.; Loew, L. M. *Biochemistry* **1989**, *28*, 4536−4539.
(5) Demchenko, A. P.; Klymchenko, A. S.; Pivovarenko, V. G.; Ercelen, S. In *Fluorescence Spectroscopy, Imaging and Probes − New Tools in Chemical, Physical and Life Sciences*; Kraayenhof, R., Visser, A. J. W. G., Gerritsen, H. C., Eds.; Springer Series on Fluorescence Methods and Applications; Springer-Verlag: Heidelberg, Germany, 2002; Vol. 2, pp 101−110.
(6) Sengupta, P. K.; Kasha, M. *Chem. Phys. Lett.* **1979**, *68*, 382−385.

10.1021/ja027669l CCC: $22.00

are represented by highly fluorescent and well-separated (up to 7000 cm^{-1}) emission bands. Positions and the intensity ratio of these bands are highly sensitive to the surrounding of the 3HF chromophore. The perturbations by intermolecular hydrogen bonding[7] and variations of solvent polarity[8] have been extensively studied, which provided the background for intensive applications of these dyes as polarity-sensitive probes in analytical chemistry,[9] polymer science,[10] colloid chemistry,[11] and biochemistry.[12] Meanwhile, the studies of their electrochromic properties were limited to the determination of the excited-state dipole moments.[13] No one addressed and responded to the question that is very important for most molecular sensor applications: can the electric field influence the ESIPT reaction and modulate the intensity redistribution between two N* and T* bands in emission?

To respond to this question, we synthesized and studied ionic 3HF derivatives with fixed proximal cation and nonfixed anion, as well as their neutral analogues. To prove the electrostatic nature of the observed effects, a series of experiments on screening and unscreening of the introduced charge was performed. We observe that the introduction of proximal charge results not only in the shifts of both N* and T* bands due to internal Stark effect[14] but also in modulation by this effect of the ESIPT reaction itself. The latter produces a dramatic change of intensity ratio between these two emission bands with a strong amplification of the electrochromic effect. Thus, we demonstrate that the ionic 3HF derivatives may serve the prototypes of electric field molecular sensors with extreme sensitivity.

Materials and Methods

Absorption spectra were recorded on a Cary 3 Bio spectrophotometer (Varian). Fluorescence measurements were recorded on a Quanta Master spectrofluorometer (Photon Technology International). Fluorescence quantum yield (ϕ) was determined using a solution of 4′-diethylamino-3-hydroxyflavone (**F**) in ethanol as the reference ($\phi = 0.52$).[8b] Deconvolution of fluorescence spectra in the cases when two bands are overlapped was made by a program Siano, kindly provided by the author (Dr. A. O. Doroshenko from the Karazin University, Kharkov, Ukraine). The program uses an iterational nonlinear least-squares method based on the Fletcher−Powell algorithm. The shapes of individual emission bands were approximated by log-normal function,[15] which accounts for the asymmetry of the spectral bands.

All solvents for absorption and fluorescence experiments were of spectroscopic grade. Hydrophobic salts tetraphenylphosphonium bromide ($[PBr_4]^+Br^-$) and sodium tetraphenylborate ($Na^+[BPh_4]^-$) were purchased from Sigma-Aldrich Chemical Co. Chloroform and ethyl acetate were chosen as the solvents for the experiments with $[PBr_4]^+Br^-$ and $Na^+[BPh_4]^-$, respectively, according to three criteria: their low dielectric constant ($\epsilon = 4.8$ and 6.0, respectively), good solubility of the corresponding salt, and well-resolved two-band emission of the studied dyes.

4′-Diethylamino-3-hydroxyflavone (**F**) was synthesized as described elsewhere.[8b] The detailed description of synthesis and purification of 6-diethylaminoethyl-4′-diethylamino-3-hydroxyflavone (**FN**), *N*-[(4′-diethylamino)-3-hydroxy-6-flavonyl]methyl-*N*,*N*-dimethyloctylammonium bromide (**FN**$^+$**Br**$^-$), 3-hydroxy-4′-[4-(4-pyridyl)piperazino]flavone (**NF**), and 4-{4-[4′-(3-hydroxyflavonyl)]piperazino}-1-(3-sulfopropyl)-pyridinium (**S**$^-$**N**$^+$**F**) will be published separately. Briefly, **FN** and **FN**$^+$**Br**$^-$ were synthesized in five steps starting from 2-hydroxyacetophenone. Its chloromethylation with paraformaldehyde in concentrated HCl at 40 °C yielded 5-chloromethyl-2-hydroxyacetophenone. The latter was condensed with 4-diethylaminobenzaldehyde affording the corresponding chalcone. Applying the common procedure,[16] we found that the latter was converted to 6-ethoxymethyl-4′-*N*,*N*-diethylamino-3-hydroxyflavone, which was transformed into 6-bromomethyl-4′-*N*,*N*-diethylamino-3-hydroxyflavone by heating at 100 °C in 62% HBr. The target flavones **FN** and **FN**$^+$**Br**$^-$ were prepared by treating the bromomethyl derivative with diethylamine and *N*,*N*-dimethyloctylamine, respectively. The flavone **NF** was synthesized also applying the common procedure[16] from 4-[4-(4-pyridyl)piperazino]benzaldehyde (prepared in two steps from 4-chloropyridine and 1-phenylpiperazine followed by introduction of the formyl group into the intermediate, 1-phenyl-4-(4-pyridyl)piperazine) and 2-hydroxyacetophenone. The latter was transformed to **S**$^-$**N**$^+$**F** with 1,3-propanesultone. The structure and purity of all of the flavones were confirmed with ^{1}H NMR and mass spectra.

6-Diethylaminomethyl-4′-diethylamino-3-hydroxyflavone (FN). mp 115−116 °C. ^{1}H NMR (200 MHz, $CDCl_3$): 1.053 (6H, t, *J* 7.1 Hz), 1.23 (6H, t, *J* 7.1 Hz), 2.54 (4H, q, *J* 7.1), 3.45 (4H, q, *J* 7.1 Hz), 3.66 (2H, s), 6.77 (2H, d, *J* 9.1 Hz), 6.89 (1H, s), 7.51 (1H, d, *J* 8.7 Hz), 7.72 (1H, dd, *J* 8.7 Hz; 1.8 Hz), 8.1 (1H, d, *J* 1.8 Hz), 8.166 (2H, d, *J* 9.1 Hz). MS (EI): *m*/*z* 394.2 (M^+), 379.2, 322.1, 307.1, 294.1, 278.1, 266.1, 250.1, 182.1, 153.5, 133.0, 86.1.

***N*-[(4′-Diethylamino)-3-hydroxy-6-flavonyl]methyl-*N*,*N*-dimethyloctylammonium Bromide (FN$^+$Br$^-$).** mp 153−154 °C. ^{1}H NMR (200 MHz, $CDCl_3$): 0.85 (3H, t, *J* 5.80), 1.2−1.4 (18H, multiplet), 3.33 (6H, s), 3.4−3.6 (6H, multiplet), 5.28 (2H, s), 6.7−7.0 (3H, multiplet), 7.59 (1H, d, *J* 8.44), 8.15 (2H, d, *J* 8.17), 8.22 (1H, s), 8.35 (1H, d, *J* 8.44). MS (FAB): *m*/*z* 479.2 (M^+), 322.1, 239.6, 154.0.

3-Hydroxy-4′-[4-(4-pyridyl)piperazino]flavone (NF). mp 264 °C. ^{1}H NMR (200 MHz, $CDCl_3$): 3.54 (8H, m), 6.71 (2H, d, *J* 6.4 Hz), 7.04 (2H, d, *J* 9.1 Hz), 7.40 (1H, m), 7.57 (1H, d, *J* 7.8), 7.68 (1H, m), 8.23 (2H, d, *J* 9.1 Hz), 8.24 (1H, d, *J* 9.5 Hz), 8.32 (2H, d, *J* 6.4 Hz). MS (EI): *m*/*z* 399.1 (M^+), 371.1, 342.1, 292.1, 264.0, 251.0, 237.0, 149.0, 106.0, 91.0, 69.1.

4-{4-[4′-(3-Hydroxyflavonyl)]piperazino}-1-(3-sulfopropyl)pyridinium (S$^-$N$^+$F). mp 270−275 °C (decomp.). ^{1}H NMR (200 MHz, $CDCl_3$): 2.11 (2H, m), 2.37 (2H, t, *J* 7.0 Hz), 3.57 (4H, t, *J* 5.6 Hz), 3.89 (4H, t, *J* 5.6 Hz), 4.32 (2H, t, *J* 6.7 Hz), 7.09 (2H, d, *J* 9.0 Hz), 7.26 (2H, d, *J* 7.4 Hz), 7.44 (1H, ddd, *J* 8.2, 6.1, 1.8 Hz), 7.61 (1H, d, *J* 8.4 Hz), 7.76 (1H, m), 8.09 (1H, d, *J* 8.2 Hz), 8.16 (2H, d, *J* 9.0), 8.35 (2H, d, *J* 7.4 Hz). MS (FAB): *m*/*z* 522.0, 400.0, 232.

(7) (a) McMorrow, D.; Kasha, M. *J. Phys. Chem.* **1984**, *88*, 2235−2243. Strandjord, A. J. G.; Courtney, S. H.; Friedrich, D. M.; Barbara, P. F. *J. Phys. Chem.* **1983**, *87*, 1125−1133. (b) Kasha, M. *J. Chem. Soc., Faraday Trans. 2* **1986**, *82*, 2379−2392. (c) Catalan, J.; Del Valle, J.-C.; Diaz, C.; Palomar, J.; De Paz, J. L. G.; Kasha, M. *Int. J. Quantum Chem.* **1999**, *72*, 421−438.

(8) (a) Swiney, T. C.; Kelley, F. D. *J. Chem. Phys.* **1993**, *99*, 211−221. (b) Chou, P.-T.; Martinez, M. L.; Clements, J. H. *J. Phys. Chem.* **1993**, *97*, 2618−2622. (c) Ormson, M. S.; Brown, R. G.; Vollmer, F.; Rettig, W. *J. Photochem. Photobiol., A* **1994**, *81*, 65−72.

(9) (a) Roshal, A. D.; Grigorovich, A. V.; Doroshenko, A. O.; Pivovarenko, V. G.; Demchenko, A. P. *J. Phys. Chem.* **1998**, *102*, 5907−5914. (b) Ercelen, S.; Klymchenko, A. S.; Demchenko, A. P. *Anal. Chim. Acta* **2002**, *464*, 273−287.

(10) Dharia, J. R.; Johnson, K. F.; Schlenoff, J. B. *Macromolecules* **1994**, *27*, 5167−5172.

(11) (a) Sakar, M.; Sengupta, P. K. *Chem. Phys. Lett.* **1991**, *179*, 68−72. (b) Klymchenko, A. S.; Demchenko, A. P. *Langmuir* **2002**, *18*, 5637−5639.

(12) (a) Bondar, O. P.; Pivovarenko, V. G.; Rowe, E. S. *Biochim. Biophys. Acta* **1998**, *1369*, 119−130. (b) Sytnik, A.; Gormin, D.; Kasha, M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11968−11972. (c) Duportail, G.; Klymchenko, A. S.; Mely, Y.; Demchenko, A. P. *FEBS Lett.* **2001**, *508*, 196−200.

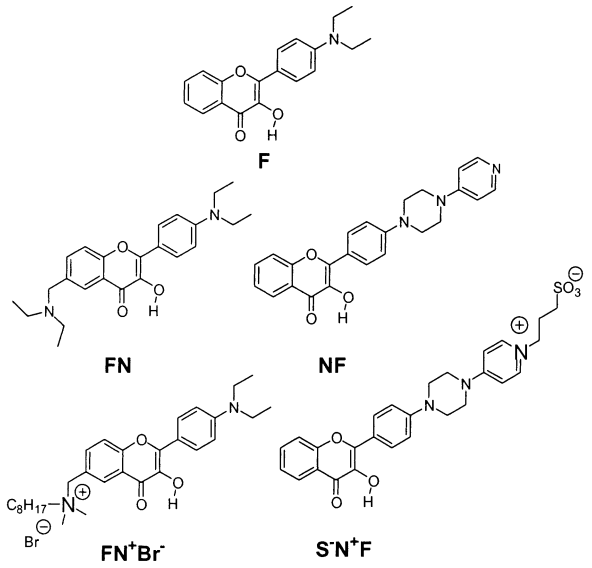
(13) (a) Premvardhan, L. L.; Peteanu, L. A. *J. Phys. Chem. A* **1999**, *103*, 7506−7514. (b) Nemkovich, N. A.; Kruchenok, J. V.; Rubinov, A. N.; Pivovarenko, V. G.; Baumann, W. *J. Photochem. Photobiol., A* **2001**, *139*, 53−62. (c) Estiu, G.; Rama, J.; Pereira, A.; Cachau, R. E.; Ventura, O. N. *J. Mol. Struct. (THEOCHEM)* **1999**, *487*, 221−230.

(14) (a) Liptay, W. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 177−188. Liptay, W. In *Excited States*; Lim, E. C., Ed.; Academic Press: New York, 1974; pp 129−229. (b) Bublitz, G. U.; Boxer, S. G. *Annu. Rev. Phys. Chem.* **1997**, *48*, 213−242.

(15) Siano, D. B.; Metzler, D. E. *J. Chem. Phys.* **1969**, *51*, 1856−1861.

(16) Smith, M. A.; Neumann, R. M.; Webb, R. A. *J. Heterocycl. Chem.* **1968**, *5*, 425−426.

Chart 1. Structures of Studied 3-Hydroxyflavones



Results and Discussion

Demonstration of Internal Stark Effect. The effect of proximal charge on the spectroscopic behavior of the 3HF chromophore was studied using absorption and steady-state fluorescence spectroscopy in a series of representative organic solvents. We designed a molecule which contains a positively charged ammonium group attached to a 3HF chromophore by a methylene bridge at the 6-position. This allows one to follow the effect of charge located at a definite position with minimal modification of the chromophore electronic structure. The target derivative **FN**$^+$**Br**$^-$ was studied in comparison with its neutral analogues **F** and 6-diethylaminomethyl-substituted derivative **FN** (Chart 1).

In all of the studied solvents, **FN**$^+$**Br**$^-$ shows absorption and fluorescence spectra shifted to longer wavelengths with respect to its neutral analogues **FN** and **F** (Table 1). Thus, in low-polar chloroform, the observed red shifts of absorption and N* emission maxima are relatively large, 1110 and 980 cm^{-1}, respectively, while the red shift of the T* band is somewhat smaller, 550 cm^{-1}. These red shifts for **FN**$^+$**Br**$^-$ are observed in all of the studied solvents (Table 1). Meanwhile, for **FN**, the positions of absorption and fluorescence bands maxima are almost the same as those of **F**. Introduction of the proximal positive charge also increases significantly the fluorescence quantum yield (ϕ). Thus, for **FN**$^+$**Br**$^-$, in all of the studied solvents ϕ is more than 2 times larger than that for its neutral analogues, reaching 0.75 in chloroform. This is the record value reported for 3-hydroxyflavones so far.

The most important spectroscopic characteristics of 3HF as the ESIPT system is the ratio of intensities of N* and T* bands in the steady-state emission spectra, I_{N*}/I_{T*}. The attached positively charged ammonium group dramatically changes this ratio (Figure 1). In all of the studied solvents, flavone **FN**$^+$**Br**$^-$ demonstrates much higher I_{N*}/I_{T*} values than those observed for its neutral analogues **F** and **FN** (Table 1). Thus, in chloroform they are 5.3- and 6.9-fold larger as compared to those of **F** and **FN**, respectively. Meanwhile, **FN**, the neutral analogue of **FN**$^+$**Br**$^-$, in comparison with **F** shows decreased I_{N*}/I_{T*} ratios.

Table 1. Spectral Characteristics of Studied 3-Hydroxyflavone Derivatives[a]

solvent	dye	λ_{max}^{abs}	λ_{max}^{N*}	λ_{max}^{T*}	I_{N*}/I_{T*}	ϕ
toluene	**F**	408	455	566	0.044	0.14
$\epsilon = 2.4$	**FN**	409	455	566	0.030	0.17
	FN$^+$**Br**$^-$	423	479	582	0.302	0.24
$CHCl_3$	**S**$^-$**N**$^+$**F**	378	455	546	0.148	0.11
$\epsilon = 4.8$	**NF**	382	467	551	0.238	0.18
	F	414	482	562	0.687	0.19
	FN	414	488	562	0.528	0.28
	FN$^+$**Br**$^-$	434	506	580[b]	3.63[b]	0.75
acetone	**S**$^-$**N**$^+$**F**	380	492	568	0.494	0.04
$\epsilon = 20.4$	**NF**	381	502	571	0.566	0.04
	F	404	502	574	0.936	0.04
	FN	403	501	574	0.564	0.05
	FN$^+$**Br**$^-$	416	521	591[b]	3.93[b]	0.17
CH_3CN	**S**$^-$**N**$^+$**F**	380	500	565	0.669	0.08
$\epsilon = 35.9$	**NF**	381	510	567	0.856	0.06
	F	404	509	571	1.76[b]	0.09
	FN	404	509	572	1.25[b]	0.10
	FN$^+$**Br**$^-$	417	528	591[b]	4.89[b]	0.32
DMSO	**S**$^-$**N**$^+$**F**	388	508	578	2.0[b]	0.13
$\epsilon = 46.5$	**NF**	388	515	578	2.1[b]	0.14
	F	411	514	584	2.83[b]	0.13
	FN	411	512	585	1.38	0.12
	FN$^+$**Br**$^-$	418	528	600[b]	4.48[b]	0.32

[a] λ_{max}^{abs}, position of absorption maxima; λ_{max}^{N*} and λ_{max}^{T*}, position of fluorescence maxima of N* and T* forms. ϕ is the fluorescence quantum yield. [b] The value was evaluated from results of the deconvolution.

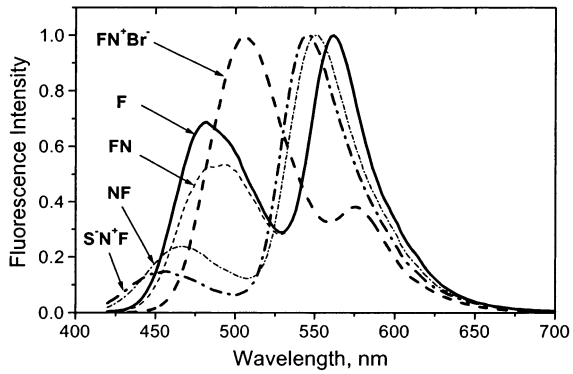


Figure 1. Fluorescence spectra of studied flavones in chloroform. The spectra are normalized at the maxima. Excitation wavelength 410 nm.

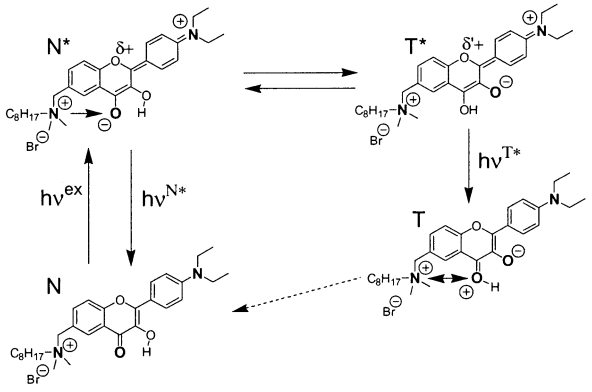
Thus, we demonstrate the influence on positions of absorption and emission bands of the positively charged group that does not participate in π-electronic conjugation with flavone chromophore. This is a clear manifestation of the internal Stark effect. We also observe a dramatic change of relative intensities of the two emission bands, which is the new fact that needs explanation.

The electrochromic shifts of spectra are the result of interaction of the chromophore ground- and excited-state dipoles with electric field produced by the proximal charge.[14] In the simplest dipole approximation, the direction and magnitude of the shift $\Delta\nu_{obs}$ are proportional to the change of dipole moment associated with the spectroscopic transition $\Delta\vec{\mu}$ and the electric field vector $\vec{F}$:

$$h\Delta\nu_{obs} = -(1/\epsilon_{ef})|\Delta\vec{\mu}||\vec{F}|\cos\theta$$

where θ is the angle between $\Delta\vec{\mu}$ and $\vec{F}$ vectors, and ϵ_{ef} is the coefficient that accounts for dielectric screening and is a microscopic analogue of dielectric constant ϵ.

Scheme 1. Four-Level Diagram of ESIPT Reaction in Ionic 3HF Derivative **FN**$^+$**Br**$^-$ with Indication of Distribution of Charges and Interactions between Them



The distribution of charges in **FN**$^+$**Br**$^-$ molecule in ground and excited states, which is based on the previous studies of 3-hydroxyflavones,[6,7,13] is shown in Scheme 1. On electronic excitation to the initial Franck−Condon state, the charge separation in the chromophore increases so that the negative charge becomes localized on the 4-carbonyl group, while the positive charge is distributed between the 1-oxygen heteroatom and 4′-amino group. The emissive N* state of this chromophore should also possess a significant charge separation, which is evident from its large solvatofluorochromy[8] and is supported by direct measurements of its dipole moment.[13b] Therefore, the proximal positive charge of **FN**$^+$**Br**$^-$ located close to 4-carbonyl stabilizes both the Franck−Condon and the N* excited states due to electrostatic interaction, shifting the absorption and N* emission bands to the red. The significant increase of the fluorescence quantum yield of **FN**$^+$**Br**$^-$ with respect to its neutral analogues is an additional indication for the strong stabilization of the N* excited state. The ESIPT reaction changes substantially the distribution of charges in the chromophore so that the negative charge migrates against the field of the proximal charge to the oxygen of the 3-OH group (Scheme 1). Therefore, the electrostatic stabilization of the T* state in **FN**$^+$**Br**$^-$ is probably absent. Unlike the N ground state, the T ground state is characterized by a considerable charge separation, and this fact should be taken into account in analysis of the shifts of the T* emission band. The charge distribution of the T state produces a dipole oriented against the field of the proximal charge, which should increase the energy of this state. This can explain the observed red shift of the T* band for **FN**$^+$**Br**$^-$. The positions of absorption and emission bands of **FN** are almost identical to those of **F**, so we can derive that the substitution at the 6-position itself does not contribute to the observed red shifts for **FN**$^+$**Br**$^-$.

Unlike the spectral shifts, the changes in relative intensities of N* and T* bands depend directly on relative energies of the corresponding states. This is the result of a very fast ESIPT reaction,[17] so that the major part of emission occurs after a dynamic equilibration between these states is reached.[8a] In **FN**$^+$**Br**$^-$, the proton has to be transferred against the performed electric field. This condition should definitely change the relative energies of the emissive states, so that the N* state becomes more stabilized (attains lower electronic energy) than the T* state. The selective stabilization with the proximal charge of N* state should result in a dramatic increase of its relative intensity, I_{N^*}/I_{T^*}. This is exactly observed for **FN**$^+$**Br**$^-$ in comparison to its neutral analogues **F** and **FN**. In contrast, a small decrease of I_{N^*}/I_{T^*} found for **FN** in comparison with **F** occurs probably because its tertiary amino group provides additional polarization of solvent molecules surrounding the 3HF chromophore, which decreases the dielectric stabilization of the latter. To exclude this possible effect, all further comparisons will be made between **FN**$^+$**Br** and its nonsubstituted neutral analogue **F**.

Additional proofs for the electrostatic origin of strong effects produced by charged substituents can be provided by the following experiments on modulation of electric field strength. (1) The positively charged group is introduced from the opposite side of the chromophore to change the direction of the electric field. (2) The dielectric constant is changed by solvent variations. (3) The proximal positive charge in **FN**$^+$**Br**$^-$ is screened by addition of hydrophobic salt composed of small bromide anion and large tetraphenylphosphonium cation. (4) The decrease of screening of proximal positive charge in **FN**$^+$**Br**$^-$ is provided by substitution of its bromide counterion for large tetraphenylborate anion.

Reversing the Directionality of Electric Field. We designed a 3HF derivative **S**$^-$**N**$^+$**F** bearing a positively charged pyridinium group fixed at the 4′-amino group, and a counterion connected by a flexible 1,3-propylene bridge (Chart 1). The neutral analogue **NF** shows considerable differences in absorption and fluorescence properties from that of parent **F** (Table 1), which is probably due to an electronic effect of chemical substitution. Therefore, the comparison is made between **S**$^-$**N**$^+$**F** and **NF**. In contrast to effects observed with **FN**$^+$**Br**$^-$, the **S**$^-$**N**$^+$**F** dye with its positive charge fixed at the opposite side of the flavone moiety demonstrates clearly the blue shifts in absorption and fluorescence spectra (Table 1). Meanwhile, the reversal of the shift effect allows one to reveal some important peculiarities. The introduced charge produces relatively small blue shifts of absorption spectra and T* emission band. These shifts are considerable (280 and 170 cm^{-1}, respectively) only in chloroform, the solvent with a low dielectric constant. Meanwhile, the blue shift observed with the N* band is large in most of the studied solvents (Table 1), attaining in chloroform a maximal value, 570 cm^{-1}. A significant change is observed for the fluorescence intensity ratio, I_{N^*}/I_{T^*}. An almost 2-fold decrease of this ratio is found in chloroform, which is also opposite to the proximal charge effect observed with dye **FN**$^+$**Br**$^-$ (see Figure 1 and Table 1).

Thus, we demonstrate that the introduction of the fixed positively charged group from the opposite side of the chromophore results in the opposite spectroscopic effects. We should emphasize that unlike the substitution of diethylamino for the 4-(4-pyridyl)piperazino group in the case of **NF**, formation of the salt in the case of **S**$^-$**N**$^+$**F** causes electrochromic but not electronic effects on the spectra. This follows from the fact that those two chemical modifications cause different spectral changes. In most of the studied solvents, for **NF** as compared to **F** a strong blue shift in absorption is not accompanied by the shift of the N* emission band, while for the case of **S**$^-$**N**$^+$**F**

(17) (a) Schwarz, B. J.; Peteanu, L. A.; Harris, C. B. J. *J. Phys. Chem.* **1992**, *96*, 3591−3598. (b) Ameer-Beg, S.; Ormson, S. M.; Brown, R. G.; Matousek, P.; Towrie, M.; Nibbering, E. T. J.; Foggi, P.; Neuwahl, F. V. R. *J. Phys. Chem. A* **2001**, *105*, 3709−3718. (c) Bader, A. N.; Ariese, F.; Gooijer, C. *J. Phys. Chem. A* **2002**, *106*, 2844−2849.

as compared to **NF** the blue shift in absorption is almost negligible with respect to the N* band (Table 1).

Here, we also have to note that the sulfonate counterion in $\mathbf{S^-N^+F}$, similar to bromide in $\mathbf{FN^+Br^-}$, should provide some screening of the positive charge. Yet this screening cannot be total, because, unlike the counterion, the positive charge is fixed to the chromophore and also because both bromide and sulfonate counterions are much larger in size than the corresponding cationic groups.

Importantly, this effect of charge located at the opposite side from the chromophore is not simply reversal. The much stronger blue shift observed with the N* emission band shows that the proximity of the positive charge to the 4′-amino group destabilizes selectively the normal emissive N* state, whereas the Franck−Condon and the T* states remain almost unaffected. This observation is also in line with the effect of charge on the I_{N*}/I_{T*} ratio, which also shows that the N* state exhibits a decreased relative intensity of emission, being probably more destabilized than the T* state. The observed disparity in spectroscopic behavior of the absorption and emission bands reveals a significant difference in the electronic structure of the corresponding excited states. Because the emissive N* state, unlike the Franck−Condon or T* state, is destabilized with the introduction of proximal charge, it can be considered as the state with significant electronic charge transfer from the 4′-amino group to the 4-carbonyl. As a result, its excited-state dipole orients against the electric field created by proximal charge. In contrast, both the initially excited and the T* states involve probably the charge transfer not from the 4′-amino group but from the 1-oxygen heteroatom. The latter is known for the nonsubstituted parent 3HF.[6,7] Therefore, the dipoles of the Franck−Condon and T* excited states should have a smaller value and different orientation than that of the N* state. This important dissimilarity of the excited states of 4′-dialkylamino-3-hydroxyflavones is in line with the fact that the absorption and T* emission bands do not show considerable solvatochromism, while the N* band is strongly solvatochromic.[8] This fact of different orientations of dipoles has never been demonstrated before in a direct electrochromic experiment.

Variation of Dielectric Constant. An increase in solvent dielectric constant ϵ should screen the electric field produced by the attached charged group. Therefore, the general expected effect is the decrease with the increase in ϵ of spectroscopic differences between the charged and neutral 3HF derivatives. This is observed in our experiment (Table 1). Thus, for ionic flavone $\mathbf{FN^+Br^-}$ with respect to neutral **F**, the red shifts in absorption and N* emission maxima decrease with the increase of ϵ (Figure 2A). Meanwhile, the red shift of the T* band does not show any correlation with ϵ, probably because, unlike the shifts in absorption and N* emission maxima, it is a result of destabilization of the T ground state (Scheme 1). In this case, the stabilizing effect on this state produced by easily polarizable bromide anion can be efficient. The ϵ increase may produce the increase in solvation of this anion, which will contribute to additional destabilization of the T state and, therefore, to compensation of the ϵ effect. In the case of $\mathbf{S^-N^+F}$, with respect to its neutral analogue **NF** the blue shifts in absorption and both emission maxima conform to a general tendency, a significant decrease of differences between their spectra with the increase of ϵ (Figure 2B). It should be added that all of the studied

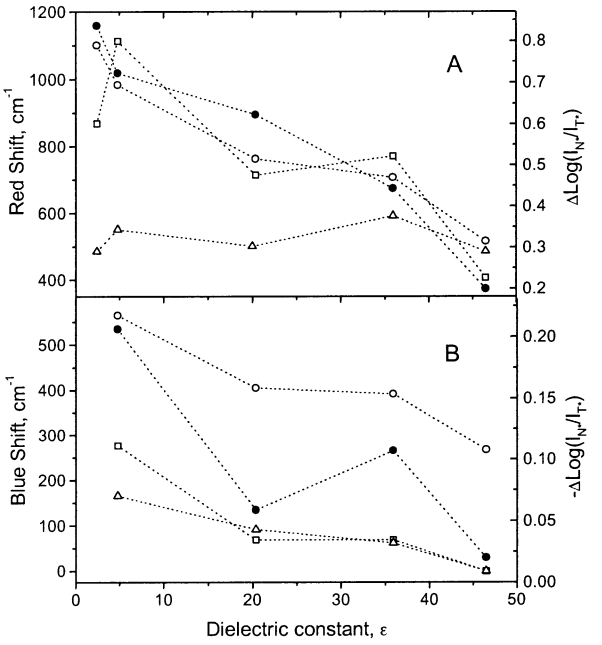


Figure 2. The effects of solvent dielectric constant (ϵ) on the difference in spectroscopic properties between charged flavones and their neutral analogues. (A) The red shifts in absorption (□), N* (○), and T* (Δ) emission bands of flavone $\mathbf{FN^+Br^-}$ with respect to **F**. (B) The corresponding blue shifts for flavone $\mathbf{S^-N^+F}$ with respect to **NF**. The corresponding differences of $\log(I_{N*}/I_{T*})$ values (●) are plotted with the scale to the right. The solvents used for the scaling of ϵ are chloroform, acetone, acetonitrile, and dimethyl sulfoxide.

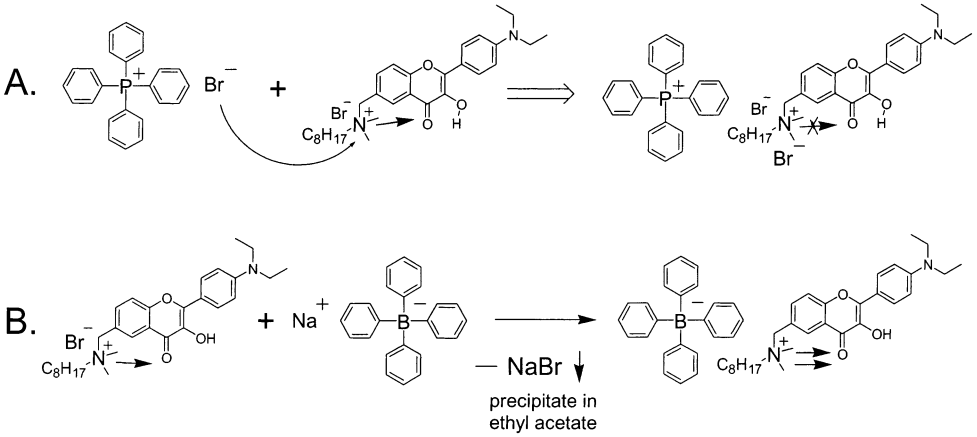
flavone dyes demonstrate solvatochromic properties typical for 3-hydroxyflavones.[7,8] With the increase of solvent polarity, the N* band maximum shifts to the red, and I_{N*}/I_{T*} increases significantly (Table 1).

An important new result is the dramatic decrease as a function of ϵ of the difference in I_{N*}/I_{T*} ratios between neutral and charged analogues. Thus, in chloroform the I_{N*}/I_{T*} values for $\mathbf{FN^+Br^-}$ and $\mathbf{S^-N^+F}$ are 5.3-fold higher and 1.6-fold lower, respectively, than those for their neutral reference compounds, while in DMSO the corresponding values differ 1.6-fold and 1.05-fold only. In view of the magnitude of this effect and with the account of its relation to the changes in relative energies of the N* and T* states, we present these ratio differences on a logarithmic scale[9b] as $\Delta\log(I_{N*}/I_{T*})$ (Figure 2).

Screening of the Proximal Charge with Counterion. We also used an alternative approach for screening the proximal charge, which is depicted in Scheme 2A. The salt $[PBr_4]^+Br^-$ when dissolved in low polar chloroform exists as an ion pair of large hydrophobic cation and small hydrophilic anion. The latter, being nonhydrated, tends to be involved in strong electrostatic interactions. Therefore, when this salt is added to dissolved $\mathbf{FN^+Br^-}$, the bromide anion should coordinate the ammonium group of the dye, the charge of which is much more localized and accessible to counterion than that of tetraphenylphosphonium cation. Therefore, with the increase of $[PBr_4]^+Br^-$ concentration, the coordination of bromide anion with the ammonium group of $\mathbf{FN^+Br^-}$ should provide an effective screening of the positive charge and shift its absorption and fluorescence spectra in the direction of that for neutral analogue **F**.

This effect is observed in our experiment (Figure 3). With an increase of $[PBr_4]^+Br^-$ concentration, the gradual blue shift

Scheme 2. Screening of the Proximal Positive Charge **FN**$^+$**Br**$^-$ with Additional Br^- Using $[PPh_4]^+Br^-$ in Chloroform (A) and Unscreening of the Proximal Positive Charge in **FN**$^+$**Br**$^-$ by Substitution of Br^- Counterion for Bulky Anion Using $Na^+[BPh_4]^-$ in Ethyl Acetate (B)



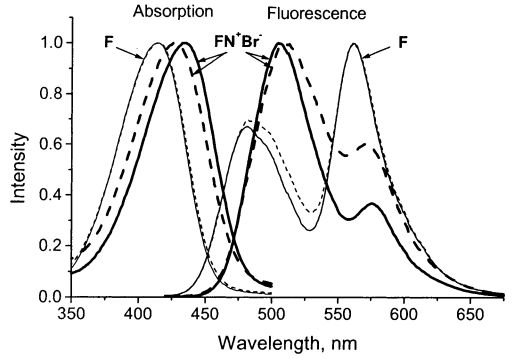


Figure 3. Absorption and fluorescence spectra of flavones **FN**$^+$**Br**$^-$ (thick lines) and **F** (thin lines) in neat chloroform (solid lines) and in a solution of 16 mM $[PBr_4]^+Br^-$ in chloroform (dashed lines). The spectra are normalized at the maxima. Excitation wavelength 410 nm.

in absorption spectra of **FN**$^+$**Br**$^-$ is observed, approaching 8 nm (430 cm^{-1}) at 40 mM (Figure 4). Meanwhile, its neutral analogue **F** shows a small red shift, which at 40 mM is only 2.5 nm (140 cm^{-1}). As a result, the difference between the absorption maxima for these two dyes decreases from 20 nm (1110 cm^{-1}) to 9.5 nm (540 cm^{-1}). In fluorescence spectra of **FN**$^+$**Br**$^-$, the considerable blue shift up to 6 nm (180 cm^{-1}) is observed with the T* band, while the N* band shows a small red shift at relatively high concentrations of the salt (Figure 3). Because a red shift of the strongly solvatochromic N* band[8] is also recorded for the neutral **F** (Figure 3), it can be assumed that addition of the salt produces some increase in the solvent polarity, which compensates its screening effect on the position of the N* band of **FN**$^+$**Br**$^-$.

The screening effect of counterions is strongly amplified when the I_{N*}/I_{T*} intensity ratio is recorded (Figures 3 and 4). With the increase in $[PBr_4]^+Br^-$ concentration, the I_{N*}/I_{T*} value decreases nonlinearly starting from 2.71 in neat chloroform and approaching 1.43 at 38.5 mM. For neutral **F** in the same conditions, a very small linear increase of this ratio is observed (Figure 4). Similar to positions of absorption maxima, the I_{N*}/I_{T*} ratios of these dyes get closer in the presence of $[PBr_4]^+Br$. The spectral effects observed with **FN** are similar to those observed with **F**, which is an additional indication that the effect of electrostatic screening is observed only in the presence of the ammonium group.

Unscreening of the Proximal Charge by Substitution of the Counterion. An attempt was also made to increase the

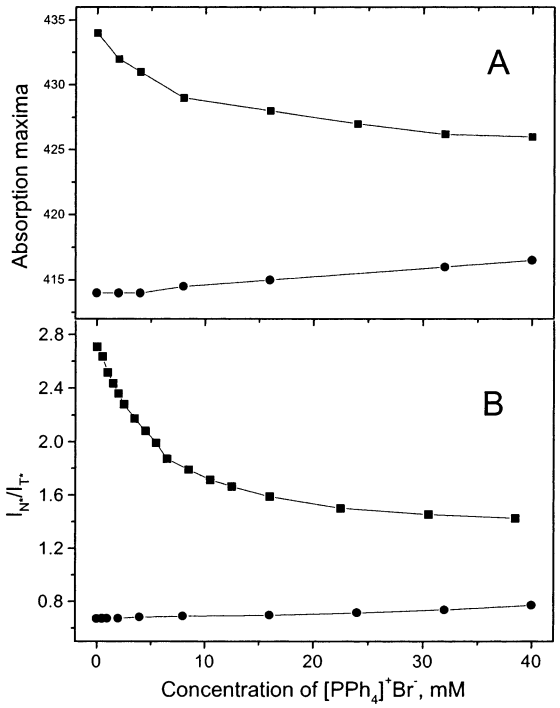


Figure 4. Positions of absorption maxima (A) and the I_{N*}/I_{T*} ratios (B) of flavones **FN**$^+$**Br**$^-$ (■) and **F** (●) in chloroform as a function of $[PBr_4]^+Br^-$ concentration.

effective positive charge of the ammonium group in **FN**$^+$**Br**$^-$. Its bromide counterion can be substituted by a large hydrophobic tetraphenylborate anion by addition of $Na^+[BPh_4]^-$ in equimolar quantities to **FN**$^+$**Br**$^-$ in ethyl acetate (Scheme 2B). The sodium cation forms salt with bromide anion, which, being nonsoluble in ethyl acetate, precipitates.

In experiments with addition of $Na^+[BPh_4]^-$ salt, the absorption and fluorescence spectra reveal two phases. Addition of the salt in concentrations below the equimolar ratio shifts to the red both absorption and emission maxima (Figure 5). In absorption spectra, the red shift increases linearly with the salt concentration and reaches the maximal value, 6 nm (350 cm^{-1}), at the point of equimolarity between the salt and the dye (Figure 6A). The same effect is also observed in emission, with the maximal values of the red shift as 11 nm (430 cm^{-1}) and 5 nm (150 cm^{-1}) for the N* and T* bands, respectively (Figure 5). At higher $Na^+[BPh_4]^-$ concentrations, a small blue (~1 nm)

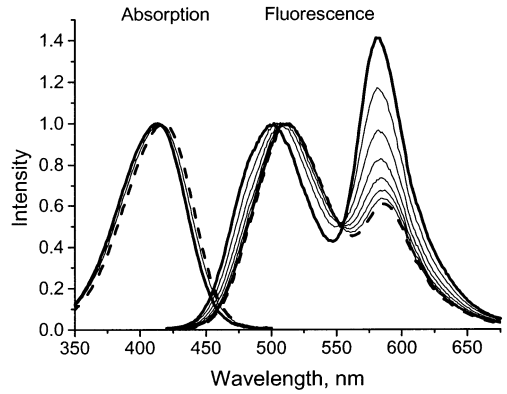


Figure 5. Absorption and fluorescence spectra of $\mathbf{FN}^{+}\mathbf{Br}^{-}$ in neat ethyl acetate (thick solid lines) and with addition of $Na^{+}[BPh_4]^{-}$ in equimolar concentration with respect to the dye, 1 μM (thick dashed lines). Intermediate points of the titration corresponding to concentration of $Na^{+}[BPh_4]^{-}$ below the equimolarity are presented as solid thin lines. The absorption and fluorescence spectra are normalized at the maxima and at N* band maxima, respectively. Excitation wavelength 410 nm.

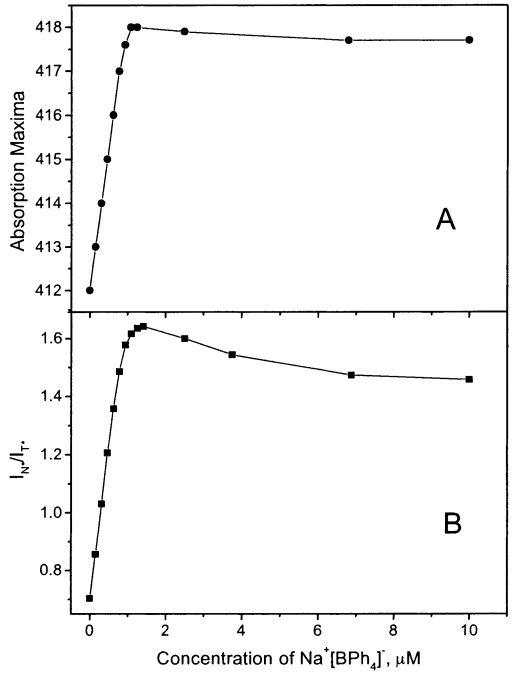


Figure 6. Positions of absorption maxima (A) and the I_{N*}/I_{T*} ratios (B) of a 1 μM solution of $\mathbf{FN}^{+}\mathbf{Br}^{-}$ in ethyl acetate upon titration with hydrophobic salt $Na^{+}[BPh_4]^{-}$.

shift in absorption and emission is observed with saturation of the effect at the salt/dye ratio over 10/1.

In these conditions, a very strong effect of variation of the I_{N*}/I_{T*} ratio is recorded (Figure 6B). Below the equimolar concentration ratio, the addition of $Na^{+}[BPh_4]^{-}$ results in a linear growth of I_{N*}/I_{T*}, which also reaches maximal values at the point of equimolarity. In this range, the I_{N*}/I_{T*} ratio increases more than 2-fold. Further addition of the salt produces the opposite effect: a smaller decrease of I_{N*}/I_{T*}, which also saturates at a salt/dye concentration ratio over 10/1. In this concentration range (0−10 μM), neither absorption nor fluorescence effects are observed with the neutral dyes **F** and **FN**.

The observed spectroscopic effects at the $Na^{+}[BPh_4]^{-}$ concentrations below the equimolarity are in full agreement with the proposed model of unscreening of the proximal positive charge in $\mathbf{FN}^{+}\mathbf{Br}^{-}$ (Scheme 2B). The substitution of the counterion is an irreversible reaction; therefore, the spectroscopic effects should increase linearly with the increase of salt concentration and then reach maximal values at the point of equimolarity. Moreover, substitution for the large counterion, which obviously screens ammonium cation with less efficiency, increases the internal Stark effect of the positive charge on the 3HF chromophore from the side of the 4-carbonyl group. This results in the red shifts in absorption and emission spectra, similar to the effects observed with the introduction of this positive charge. The increase of charge perturbation selectively stabilizes the N* state with respect to T* state, which results in a dramatic increase of its relative intensity, I_{N*}/I_{T*}. This is an additional demonstration of the coupling of relatively small electrochromic spectral shifts with strong modulation of the ESIPT reaction, which provides a dramatic change of the I_{N*}/I_{T*} ratio. The observed small blue shift and decrease in I_{N*}/I_{T*} ratio with further increase of the hydrophobic salt concentration are probably a result of dielectric screening of the positive charge.

Presented in Figures 5 and 6, results of titration by the salt of hydrophobic ion illustrate one of the possibilities: how the ionic sensor based on electrochromic dye exhibiting ESIPT can work. If the binding site for the analyte ion is designed at the position of the ammonium group, the binding of this ion will result in a strong electrochromic perturbation of the ESIPT and, therefore, in dramatic ratiometric response.

Photophysics of Modulation of Excited-State Reactions by Electric Fields. Because the internal Stark effect changes the energies of electronic excited states, its action cannot be limited to the shifts of absorption and emission spectra only. The electrostatic fields can modulate dramatically the probabilities and rates of excited-state reactions. This fact is manifested clearly in the operation of the photosynthetic reaction center, where the primary charge separation in the symmetric special pair instead of being random achieves unidirectionality under the influence of electrostatic field performed in the protein matrix.[18] There are other examples of the influence of electric field on excited-state reactions: electron transfer[19] and formation of excimers and exciplexes.[20] The corresponding studies on ESIPT systems are lacking. In this respect, 3-hydroxyflavones are of special interest, because the excited-state proton transfer in this system does not result in charge separation. Even more, it occurs in the direction of compensation of charge asymmetry created in the normal excited state.[21] Because the key elementary step of this reaction is ultrafast proton tunneling,[17] the relative contribution of N* and T* forms in emission should be determined by energetic factors. This is probably the origin of the good correlation between spectral shifts and changes in I_{N*}/I_{T*} ratio that was observed in our experiments. Previously, this correlation was observed in solvatochromic studies,[8,9b] and

(18) (a) Steffen, M. A.; Lao, K.; Boxer, S. G. *Science* **1994**, *264*, 810−816. (b) Moore, L. J.; Zhou, H.; Boxer, S. G. *Biochemistry* **1999**, *38*, 11949−11960.
(19) (a) Kawabata, H.; Nishimurs, Y.; Yamazaki, I.; Iwai, K.; Ohta, N. *J. Phys. Chem. A* **2001**, *105*, 10261−10270. (b) Kawabata, H.; Ohta, N.; Arakawa, H.; Ashida, M.; Kohtani, S.; Nakagaki, R. *J. Chem. Phys.* **2001**, *114*, 7723−7726.
(20) (a) Ohta, N.; Kawabata, H.; Yamazaki, I. *Chem. Phys. Lett.* **1999**, *310*, 397−404. (b) Ohta, N.; Umeuchi, S.; Yamazaki, I. *Chem. Phys. Lett.* **1997**, *279*, 215−222. (c) Takehara, Y.; Ohta, N.; Shiraishi, S.; Asaoka, S.; Wada, T.; Inoue, Y. *J. Photochem. Photobiol., A* **2001**, *145*, 53−60.
(21) (a) Barbara, P. F.; Walsh, P. K.; Brus, L. E. *J. Phys. Chem.* **1989**, *93*, 29−34. (b) Douhal, A.; Lahmani, F.; Zewail, A. H. *Chem. Phys.* **1996**, *207*, 477−498.

because solvatochromic and electrochromic effects are intrinsically connected,[14a] one should expect strong electric field influence on the ESIPT reaction in this system. This fact is demonstrated in the present work.

The Prospects for Color-Changing Fluorescence Sensors Based on Electrochromism of 3-Hydroxyflavones. Presently, the commonly explored mechanisms of sensing involve direct influence on the π-electronic system or influence mediated by sensor groups that are in direct electronic conjugation with this system.[1,2] In contrast, electrochromism, which is based on long-range Coulomb interactions and does not require direct electronic coupling of the chromophore with the recognition group or the analyte, can allow tremendous possibilities in design of different sensors with desired properties. In microheterogeneous systems with nonrandom distribution of charges such as biological membranes and protein macromolecules, this can provide the possibility of designing the sensors for local charge and electrostatic potential. Indeed, the electrochromic effect has been applied in very different areas, such as the sensing of electrostatic potentials in biological membranes,[4] evaluation of electric field produced by α-helical conformation in polypeptides,[22] obtaining the information on electrostatic environments of carotenoids in photosynthetic membranes,[23] explanation of the shifts in tryptophan fluorescence as a function of protein conformation,[24] and of directionality of electron transfer in the photosynthetic reaction center.[18] The broader application of molecular sensors based on this mechanism is limited by the sensitivity of their response. The results of the present research suggest the means to break this limit. The common electrochromic effects can be coupled with the ESIPT reaction. This allows one to transform small electrochromic shifts of spectra into a strong variation of relative intensities of two well-resolved emission bands with a dramatic change of emission color.

We demonstrate that this new principle can be realized in the 3-hydroxyflavone chromophore. Because the ESIPT reaction in 3HF derivatives is so sensitive to electric field perturbations, they can be used as basic elements in molecular sensors and probes that detect the variations of electric field, binding of ions, etc. However, due to the high sensitivity of this reaction also to various solvent effects,[7,8] it will not immediately result in an increase of selectivity to electric field effects on the background of other perturbations that is important for different sensor applications. We believe that the gain of selectivity can be achieved by proper sensor design. The family of 3-hydroxychromones and 3-hydroxyflavones provides a variety of substitutions in the basic chromophore that can modify in the desired direction not only the spectral positions of absorption and emission bands, but also the range of sensitivity of ratiometric response.[25] Because of the amphiphilic nature of $\mathbf{S^-N^+F}$ and $\mathbf{FN^+Br^-}$ molecules, they can already be tested as electrochromic probes in the study of electrostatic potential in biomembranes. This work is currently in progress.

Acknowledgment. This work was supported in part by a TUBITAK MRC grant.

JA027669L

(22) Lockhart, D. J.; Kim, P. S. *Science* **1992**, *257*, 947−951.
(23) Gottfried, D. S.; Steffen, M. A.; Boxer, S. G. *Science* **1991**, *251*, 662−665.
(24) (a) Vivian, J. T.; Callis, P. R. *Biophys. J.* **2001**, *80*, 2093−2109. (b) Callis, P. R.; Burgess, B. K. *J. Phys. Chem. B* **1997**, *101*, 9429−9432.
(25) (a) Klymchenko, A. S.; Ozturk, T.; Pivovarenko, V. G.; Demchenko, A. P. *Can. J. Chem.* **2001**, *79*, 358−363. (b) Klymchenko, A. S.; Ozturk, T.; Pivovarenko, V. G.; Demchenko A. P. *Tetrahedron Lett.* **2001**, *42*, 7967−7970. (c) Klymchenko, A. S.; Ozturk, T.; Demchenko, A. P. *Tetrahedron Lett.* **2002**, *43*, 7079−7082.