Dipole Moment Change of NBD Group upon Excitation Studied Using Solvatochromic and Quantum Chemical Approaches: Implications in Membrane Research

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Lipids that are covalently labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group are widely used as fluorescent analogues of native lipids in model and biological membranes to study a variety of processes. We have recently shown that one such NBD-labeled lipid, NBD-PE, in which the NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule, exhibits the red edge excitation shift (REES) effect when incorporated into vesicles of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) [Chattopadhyay, A.; Mukherjee, S. *Biochemistry* **1993,** *32,* 3804]. One of the necessary conditions for a fluorophore to be able to exhibit REES is that the fluorophore must be polar and, more importantly, there should be a change in its dipole moment upon excitation. In this paper, we have determined the actual change in dipole moment of the NBD group upon excitation using the solvatochromic shift approach. Our results show that the dipole moment of the NBD group changes by 3.9 D upon excitation. We have complemented this experimental observation by semiempirical quantum chemical calculations of dipole moment changes of various NBD derivatives. These calculated dipole moment changes (3.5-3.6 D) agree very well with our experimental value. These calculations also point out that the process of charge separation is mainly limited to the NBD ring system and is independent of the length of the alkyl chain. These results are relevant to ongoing and future studies that utilize photophysical properties of the NBD group, especially in microheterogeneous media such as membranes and micelles.

Introduction

Fluorescence spectroscopy has been one of the principal techniques to study organization and dynamics of biological and model membranes because of its suitable time scale, noninvasive nature, and intrinsic sensitivity.[1-3] One fluorescent group that is widely used to label lipids is the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group. NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes [for a review, see ref 4]. These processes include membrane fusion, lipid motion and dynamics, organization of lipids and proteins in membranes, intracellular lipid transfer, and bilayer to hexagonal phase transition in liposomes. The fluorescence behavior of these probes have been previously characterized both in organic solvents[5-7] and in membranes.[5,6] We have recently shown that a commonly used NBD-labeled lipid, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), in which the NBD label is covalently attached to the headgroup of a phosphatidylethanolamine molecule, exhibits the phenomenon of red edge excitation shift when incorporated into vesicles of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).[8,9]

A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases. The origin of the red edge effect lies in the change in fluorophore–solvent interactions in the ground and excited states, brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent molecules reorient around the excited-state fluorophore.[10-18] An important criterion for a fluorophore to be able to exhibit REES is that it should be polar; that is, it

should have a permanent dipole moment in the ground state. In addition, there should be a change in the dipole moment upon excitation, so as to cause the solvent dipoles to reorient in response to this altered dipole moment in order to attain an energetically favorable orientation. For a totally nonpolar fluorophore, there will be no change in the dipole moment upon excitation, and the process of solvent reorientation becomes irrelevant, since it is the change in dipole moment that triggers the solvent reorientation. We have recently confirmed this[9] by monitoring the effect of changing excitation wavelength on the wavelength of maximum emission for the nonpolar fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) in DOPC vesicles. We found no change in the emission maximum of DPH as the excitation wavelength was varied over a wide range. In other words, DPH in DOPC vesicles does not exhibit any REES.

In this paper, we have determined the actual change in the dipole moment of the NBD group upon excitation using the solvatochromic shift approach.[19-23] Our results show that the dipole moment of the NBD group changes by 3.9 D upon excitation. This is further supported by calculation of dipole moment change using semiempirical quantum chemical calculations.[24] These calculations were performed on a series of model compounds all having the NBD ring system but differing in the length of the alkyl chain. Our calculations show that the dipole moment change of the model compounds upon excitation is very similar to what we obtain from solvatochromic shift method for 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (NBD-AHA) and that the length of the alkyl chain of the molecule is not critical for dipole moment changes. The structures of NBD-AHA and the NBD model compounds are shown in Figure 1.

Experimental Section

NBD-AHA was purchased from Molecular Probes (Eugene, OR). The $E_T(30)$ dye [2,6-diphenyl-4-(2,4,6-triphenyl-*N*-pyridino)phenoxide] was a kind gift from Dr. Christian Reichardt (Philipps University, Marburg, Germany). All solvents used were of spectroscopic grade, whose purity were further checked by the $E_T(30)$ procedure.[25,26]

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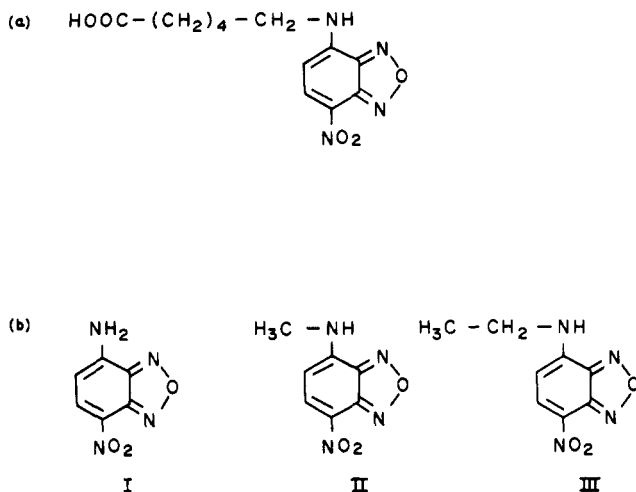
(a)

(b)

Figure 1. (a) Structure of NBD-AHA; (b) structures of NBD derivatives I-III used for calculation of dipole moment change.

The $E_T(30)$ dye is a pyridinium-N-phenoxide betaine dye which exhibits a negatively solvatochromic $\pi \rightarrow \pi^*$ absorption band with an intramolecular charge-transfer character. This molecule undergoes one of the largest known solvent-induced shifts in absorption maximum, amounting to 357 nm (9730 cm^{-1}) in going from water (453 nm) to diphenyl ether (810 nm). This extremely large solvent-induced shift of the visible $\pi \rightarrow \pi^*$ absorption band has been used to introduce an empirical parameter of solvent polarity, called the $E_T(30)$ value. According to the following equation, the $E_T(30)$ value for a solvent is defined as the transition energy of the dissolved betaine dye measured in kcal/mol:

$$E_T = hc\bar{\nu}N_A = 2.859 \times 10^{-3}\bar{\nu} \qquad (1)$$

Here, h is Planck's constant, c is the velocity of light, $\bar{\nu}$ is the wavenumber of the photon in cm^{-1} which produces the electronic transition, and N_A is Avogadro's number. Due to the extremely large solvatochromism of this dye, the $E_T(30)$ values provide an excellent characterization of the polarity of solvents and thus serve as sensitive indicators for trace amounts of water present in any solvent. $E_T(30)$ values have been previously determined for more than 270 pure solvents.[25]

For estimating the purity of our solvents, a few grains of the $E_T(30)$ dye were dissolved in the solvent of interest, and its absorption maximum was monitored. From this absorption maximum, the $E_T(30)$ value was calculated using eq 1. The $E_T(30)$ value so obtained was then compared with the values reported in the literature.[25] The $E_T(30)$ values obtained by us showed a maximum deviation of less than 0.4% from the reported values for the solvents used in this study.

NBD-AHA was used at a concentration of 20 μM in various solvents to monitor both the absorption and emission band maxima. This concentration is much lower than the concentrations at which NBD-AHA is known to aggregate.[7] Absorption spectra were recorded using a Hitachi 150-20 UV–visible absorption spectrophotometer after appropriate baseline corrections. Corrected fluorescence spectra were recorded with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Slits with a nominal band-pass of 3 nm were used for all measurements. Background intensities of pure solvents were subtracted from each sample spectrum. All experiments were done at 23 °C.

Dipole moment calculations for the NBD derivatives I-III (Figure 1b) were carried out using a standard AM1 program (AMPAC version 2.1) which consists of an improved parametrization for modified neglect of diatomic overlap (MNDO) Hamiltonian[24] on a DEIL VAX 3300 computer. The geometries of the molecules in the ground and excited states were fully optimized. For excited-state calculations, configuration inter-

TABLE 1: Solvatochromism of NBD-AHA Fluorescence

solvent	ϵ^a	n^b	$\bar{\nu}_a$ (cm^{-1})	$\bar{\nu}_f$ (cm^{-1})	$\bar{\nu}_a - \bar{\nu}_f$ (cm^{-1})
pyridine	12.3	1.507	21 186	18 797	2389
tetrahydrofuran	7.58	1.404	21 882	19 417	2465
2-propanol	18.3	1.375	21 413	18 692	2721
acetone	20.7	1.357	21 598	18 868	2730
dimethyl sulfoxide	46.45	1.476	21 097	18 349	2748
methanol	32.63	1.326	21 459	18 553	2906
trifluoroethanol	26.67	1.290	21 367	18 416	2951

^{a} From refs 25, 35 (pp E51–E53), and 36. ^{b} From ref 35 (pp E381–E383).

action (CI) was taken into account using a total of 100 configurations. Both singly and doubly excited configurations were considered.

Results

We have used NBD-AHA as the model compound for the solvatochromic experiments. This derivative was selected for its aminoalkyl side chain's similarity to the covalent linkage found in NBD-labeled lipids such as NBD-PE (as opposed to other NBD derivatives such as NBD chloride). At the same time, this molecule is small enough not to be aggregated in solvents (especially of low dielectric constant) at the concentrations used.[6,7] The absorption and emission maxima ($\bar{\nu}_a$ and $\bar{\nu}_f$, respectively) obtained for NBD-AHA in solvents of varying polarity are shown in Table 1 along with the dielectric constants (ϵ) and the refractive indices (n) of the solvents. The extent of the Stokes' shift ($\bar{\nu}_a - \bar{\nu}_f$) shows considerable sensitivity to solvent polarity, indicating that there is a significant difference between ground- and excited-state dipole moments.

Although a number of expressions have been proposed to describe the effects of solvent polarity on the emission spectra of fluorophores, the most widely used expression was derived by Lippert and other workers.[19-22] Onsager's reaction field theory provides the basic framework for the Lippert equation, which assumes the fluorophore to be a point dipole held in the center of a spherical cavity (the Onsager cavity), in a homogeneous and continuous dielectric medium.[27,28] According to this model, the fluorophore–solvent interaction is given by the interaction of the dipole at the cavity with its own reaction field arising from the polarization of the surrounding dielectric by the dipole. If the solvent reorientation is fast compared to the lifetime of the fluorophore (i.e., solvent reorientation around the excited-state fluorophore is complete prior to emission), then the dependence of Stokes' shift on the change in the dipole moment of the fluorophore upon excitation is given by the Lippert equation.[19-22] This equation represents the collective influence of the entire set of surrounding solvent molecules on the fluorophore. The solution of this equation requires the use of measurable parameters such as the Onsager cavity radius (which for practical purposes is considered equal to the van der Waals radius) of the fluorophore, the excitation and emission band maxima in solvents of varying polarities, and the dielectric constants and refractive indices of the solvents used. The basic tenet of this equation is the fact that the interactions between the solvent and fluorophore molecules affect the energy difference between the ground and excited states. To a first approximation, this energy difference (in cm^{-1}) is a property of the refractive index (n) and the static dielectric constant (ϵ) of the solvent, and is described by the Lippert equation:

$$\bar{\nu}_a - \bar{\nu}_f = \frac{2(\mu_e - \mu_g)^2}{hca^3}\left[\frac{\epsilon - 1}{2\epsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right] + \text{constant} \qquad (2)$$

In this equation, h is Planck's constant, c is the speed of light, and a is the Onsager cavity radius. The wavenumbers in cm^{-1}, of the absorption and emission bands, are designated as $\bar{\nu}_a$ and $\bar{\nu}_f$, respectively, while μ_e and μ_g refer to the dipole moments of the fluorophore in the excited and ground state. The higher order
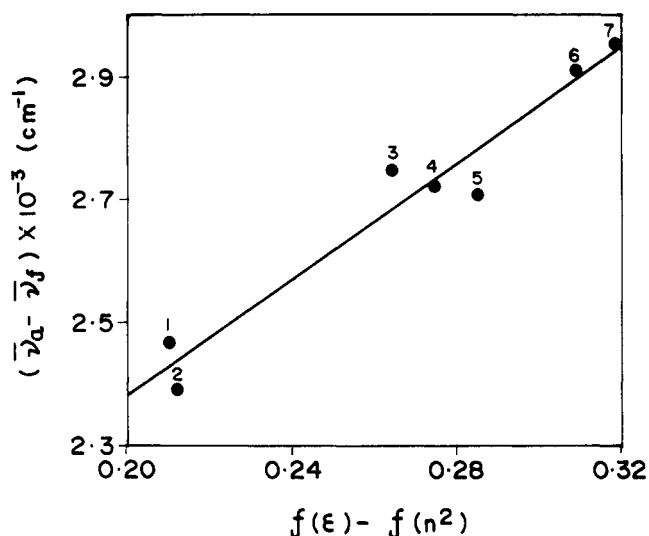
Figure 2. Dependence of Stokes' shift $(\bar{\nu}_a - \bar{\nu}_f)$ of NBD-AHA on orientation polarizability $[f(\epsilon) - f(n^2)]$. Solvents used were tetrahydrofuran (1), pyridine (2), dimethyl sulfoxide (3), 2-propanol (4), acetone (5), methanol (6), and trifluoroethanol (7). The concentration of NBD-AHA was 20 μM. See text for other details.

terms in eq 2 are due to second-order effects such as contributions of the solvent–solvent interactions to the reaction field and the induced dipole moments of the solvent molecules resulting from the excited-state dipole moment of the fluorophore. These are normally neglected since they have very small contribution.[23,30–32]

The change in the dipole moment of a fluorophore upon excitation can be calculated from the slope of the Lippert plot.[21] This is a plot of the Stokes' shift $(\bar{\nu}_a - \bar{\nu}_f)$ versus the orientation polarizability $[f(\epsilon) - f(n^2)]$. Figure 2 shows the Lippert plot for NBD-AHA in various solvents. The linearity of this plot is indicative of predominantly general solvent effects. The Onsager cavity radius for NBD-AHA was assumed to be 3.19 Å based on its energy minimized structure using the AM1 program (see Discussion). The difference between the ground and excited state dipole moments $(\mu_e - \mu_g)$ was calculated from the slope of Figure 2. A linear regression analysis ($r = 0.95$) of the plot gave a slope of 4704.6 cm^{-1} which amounts to a change in dipole moment of 3.9 D for NBD-AHA.

TABLE 2: Dipole Moments of NBD Derivatives

compound	μ_g (D)	μ_e (D)	$\Delta\mu$ (D)	method used
I	9.1	12.6	3.5	AM1 calculation
II	9.6	13.2	3.6	AM1 calculation
III	9.5	13.2	3.5	AM1 calculation
NBD-AHA^a	–	–	3.9	solvatochromic shift

^{a} Assuming an Onsager cavity radius of 3.19 Å (see text).

For dipole moment calculations using AM1 program, we chose the NBD model compounds I–III (see Figure 1b). These model compounds are identical in their chemical structure to NBD-AHA with regard to both the aromatic rings and the aminoalkyl chain, except that the length of the alkyl chain is different. We reasoned that the presence of the polar carboxyl group at one end of NBD-AHA will not make a difference since it is separated by a nonpolar polymethylene spacer along which it is unlikely to have charge separation. Results from our calculations show that this is a valid assumption (see later).

The electronic charge densities of the NBD derivatives I–III in their ground and excited states, calculated using the AM1 program, are shown in Figure 3. The dipole moments calculated from these charge densities and the differences in dipole moment between the ground and excited states for compounds I–III are shown in Table 2 along with that for NBD-AHA obtained from the Lippert plot (Figure 2). For the simplest of these compounds (I) in which the alkyl chain is simply replaced by hydrogen, the difference in dipole moment $(\Delta\mu)$ amounts to 3.5 D. As one of the hydrogens of compound I is replaced by a methyl group (compound II), both the ground- and excited-state dipole moments increase by $\sim$0.5 D. This is consistent with the inductive effect of the alkyl group which favors more charge separation between the amino nitrogen (N-11) and the oxygen atoms (O-14 and -15) in the nitro group (see Figure 3). On replacement of the methyl group by an ethyl group (compound III), the dipole moments remain more or less constant. It is indeed remarkable that, in all these cases, $\Delta\mu$ remains essentially invariant. This internal consistency increases the reliability of the calculations. In addition, it indicates that the length of the alkyl chain does not play an important role in charge separation and the resultant dipole moment. This justifies our assumption (stated above) that compounds I–III are excellent model compounds for NBD-AHA, insofar as charge separation and dipole moments are concerned.
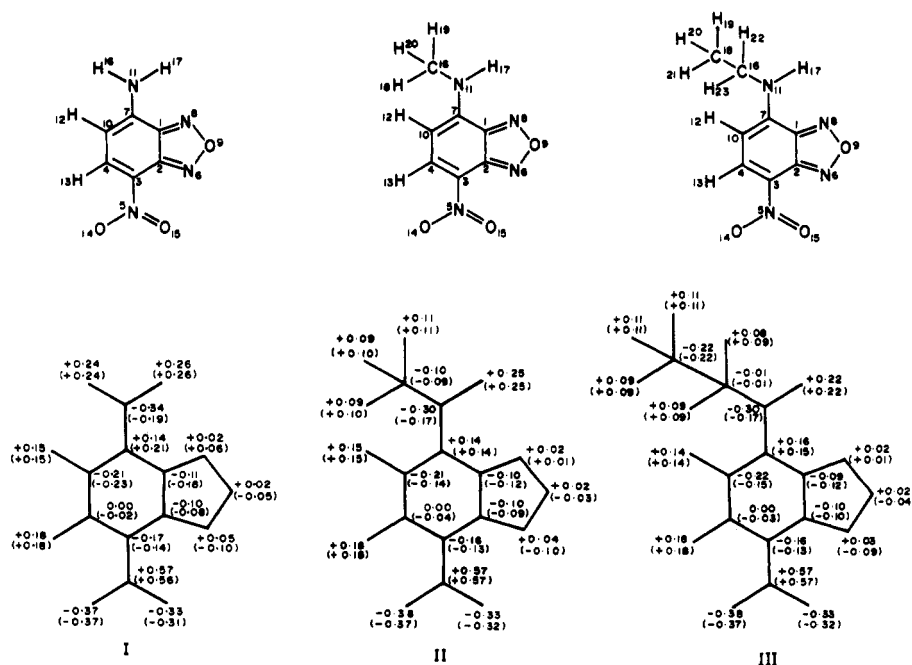

Figure 3. Electronic charge densities at the ground and excited states for the NBD derivatives I–III. The charge densities for the excited state are shown within parentheses. The charge densities were calculated using a standard AM1 program. See Experimental Section for other details.

Table 2 also compares the dipole moment changes for these model compounds by AM1 calculation and solvatochromic shift method. The value of $\Delta\mu$ obtained from the Lippert equation in case of NBD-AHA is in excellent agreement (within 10%) with the values obtained from AM1 calculations. This supports our choice of Onsager cavity radius for NBD-AHA based on the maximum distance along which charge separation takes place in the energy-minimized structure.

Discussion

The change in dipole moment of the NBD group upon excitation has been the focus of this report. Solvatochromic shift studies using NBD-AHA indicate that the change in dipole moment amounts to 3.9 D. In close agreement with this, semiempirical quantum chemical calculations using the AM1 program on a series of NBD derivatives show that the dipole moment changes by 3.5–3.6 D between the ground and excited states. This change in dipole moment of the NBD group is significant in the context of solvent reorientation in its excited state.[9]

An important parameter in the Lippert equation is the Onsager cavity radius.[29] It is operationally defined as the radius of a spherical cavity surrounding the molecule in which the molecule resides and is usually equated to its molecular (van der Waals) radius.[30-32] We have calculated the Onsager cavity radius for the NBD moiety from the energy minimized structure of the NBD derivative I using the AM1 program. The distance between the average coordinates of the oxygen atoms (O-14 and -15) of the nitro group on one end of the molecule and the nitrogen atom of the amino group (N-11) on the other end is the longest distance across the molecule where charge separation could occur (see Figure 3). This distance was found to be 6.38 Å. Therefore, the Onsager cavity radius was taken as half of this distance, *i.e.*, 3.19 Å. The hexanoic acid chain that is attached at the amino group in NBD-AHA was not taken into account while calculating the Onsager cavity radius, because no charge separation (and, consequently, no contribution to the dipole moment of the molecule) is expected across the nonconjugated alkyl chain. This is further supported by the fact that, although the longest molecular axis in the NBD derivatives I–III vary significantly, it does not appear to have any effect on the calculated value of $\Delta\mu$ (Table 2). Thus, it is obvious that one should consider, for the purpose of Onsager cavity radius, the maximum distance separating charge centers rather than the longest molecular axis. If it were not so, $\Delta\mu$ for compound III based on its longest axis would have been 5.5 D (*i.e.*, 57% more than the calculated $\Delta\mu$ of 3.5 D) since from the Lippert equation $\Delta\mu^2$ is proportional to a^3.

Any change in the dipole moment of a fluorophore upon excitation calculated from the Lippert equation will thus be critically dependent on the choice of the Onsager cavity radius. In fact, it has been previously estimated that the value obtained for dipole moment change of a fluorophore from the Lippert equation may differ from the actual value by as much as 50% depending on the choice of the Onsager cavity radius.[30,32] In our case, if we take the Onsager cavity radius for NBD-AHA to be 4.00 Å (instead of 3.19 Å), the dipole moment change upon excitation increases from 3.9 to 5.5 D. We would thus like to emphasize the need to use caution while choosing Onsager cavity radius. Furthermore, it is desirable to get an estimate of the dipole moment by other methods so as to be able to evaluate the appropriateness of choice of the Onsager cavity radius. Incorrect choice of Onsager cavity radius could lead to erroneous estimates of dipole moment changes.

The NBD group is widely used as fluorescent membrane probe.[4] The solvatochromic properties of the NBD group are useful in studies of microenvironmental polarity in membrane domains.[33,34] Our results on dipole moment changes and solvatochromism of the NBD moiety are relevant to ongoing and future studies that utilize photophysical properties of the NBD group, especially in microheterogeneous media such as membranes and micelles.

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