Long-Range Fluorescence Quenching of Ethidium Ion by Cationic Porphyrins in the Presence of DNA

Robert F. Pasternack,* Melissa Caccam, Bart Keogh, Thomas A. Stephenson, Alison P. Williams, and Esther J. Gibbs†

Contribution from the Department of Chemistry, Swarthmore College, Swarthmore, Pennsylvania 19081. *Received November 16, 1990. Revised Manuscript Received May 17, 1991*

Abstract: Binding of ethidium to DNA increases the fluorescence efficiency of the molecular ion by about 1 order of magnitude. Quenching of the fluorescence of such an ethidium·DNA complex by tetrakis(4-*N*-methylpyridyl)porphine (H_2T4), and its nickel(II) and zinc(II) derivatives (NiT4 and ZnT4), was investigated in the present study. Results of absorbance and fluorescence measurements obtained for these systems are consistent with a mechanism for quenching that does not involve appreciable displacement of ethidium from the nucleic acid. Stern–Volmer-type plots are nonlinear and can be fit satisfactorily by an equation of the form

$$\frac{I_0}{I} = \exp\left(2\sigma[Q] \Big/ \left(\left(1 - \frac{2[E^+]}{[DNA]}\right)[DNA]\right)\right)$$

in which [Q] is the concentration of the porphyrin quencher and σ is the minimum number of base pairs between ethidium (E^+) and the porphyrin required to permit the excited fluorophore to emit a photon. It can be concluded from these studies that, in the presence of DNA, porphyrins are capable of efficiently quenching the fluorescence of an excited ethidium ion at a distance of 25–30 Å.

Nucleic acid bound small molecules (often termed "ligands" or "drugs") generally show marked changes in absorbance and, where relevant, fluorescence properties as compared to their spectral characteristics when free in solution. Ethidium ion (E^+; Figure 1), for example, displays a bathochromic shift and hypochromicity of its visible absorption band as well as a dramatic enhancement of its fluorescence efficiency when complexed to DNA.[1,2] A pathway for *nonradiative* decay of excited ethidium ($^*E^+$) in aqueous solution has been proposed in which the ion donates a proton from one of its amino groups to the solvent.[3] However, when intercalated into DNA[2,4] and thereby isolated from the solvent, this proton-transfer pathway is virtually eliminated, leading to a lengthening of the ethidium excited-state lifetime to 23 ns (from 1.8 ns in water) and an increase in the molar fluorescence intensity by about 1 order of magnitude (Figure 2).[1,2,5,6]

It has been previously reported that this enhanced fluorescence of the ethidium·DNA complex can be quenched at least partially by the addition of a second drug molecule.[4,6-9] Ligand–ligand interactions of this type are not limited to ethidium systems—a variety of cobalt(III) polypyridyl complexes have been shown, in the presence of DNA, to be efficient quenchers of $Ru(phen)_3^{2+}$ and $Ru(bipy)_3^{2+}$ fluorescence.[10] Two mechanisms have been proposed to account for quenching: displacement of molecular ion fluorophores and/or electron transfer.[4,6-10] An experimental strategy for determining stability constants for "unknown" drug molecules based upon quenching of ethidium fluorescence via competition for binding sites has become a standard method in nucleic acid chemistry.[4] However, caution must be exercised when applying this approach because efficient quenching has also been demonstrated for several systems when ethidium and the test molecule are *simultaneously* bound to DNA.[6-9,11] This non-displacement-based quenching has been correlated with DNA-enhanced electron transfer, either from excited ethidium to an acceptor[6,9] (methylviologen, MV^{2+}, or cupric ion, Cu^{2+}) or from an amsacrine donor to an excited ethidium acceptor.[8] Similarly, cobalt(III) polypyridyl quenching of ruthenium(II) complexes is purported to proceed via electron transfer.[10] The influence of DNA is quite dramatic in these quenching pathways. With MV^{2+} as acceptor and ethidium as donor,[6] the nucleic acid matrix is reported to enhance the yield of electron-transfer events by a factor of 5×10^5; the rate of electron transfer between $^*Ru(phen)_3^{2+}$ and $Co(phen)_3^{3+}$ is catalyzed by DNA to the extent of about 2 orders of magnitude.[10] A number of factors have been proposed as contributing to these quenching efficiencies: increase in fluorescence lifetime of the donor, local concentration effects arising from simultaneous binding of donor and acceptor molecules to the polymer, and reduced dimensionality at the polymer surface, as it pertains to reactant mobility.[6,10]

The present study complements and extends previous reports on donor–acceptor communication as facilitated by DNA, and probes several new areas. We describe here the quenching of ethidium ion fluorescence by several water-soluble cationic porphyrins that have been shown to interact extensively with DNA.[12-14] In particular, tetrakis(4-*N*-methylpyridyl)porphine (H_2T4; Figure 1) has been demonstrated to intercalate into calf thymus (ct) DNA under low ionic strength conditions but to convert to an exterior, groove-binding mode as salt concentration is increased.[15] In contrast, at low drug load, the nickel(II) derivative of this porphyrin (NiT4) binds to ct DNA exclusively as an intercalator[13,14,16] whereas ZnT4, which contains one axial ligand, binds externally, presumably in the minor groove.[13,14,17]

* To whom inquiries should be addressed.
† Department of Chemistry, Goucher College, Towson, MD 21204.

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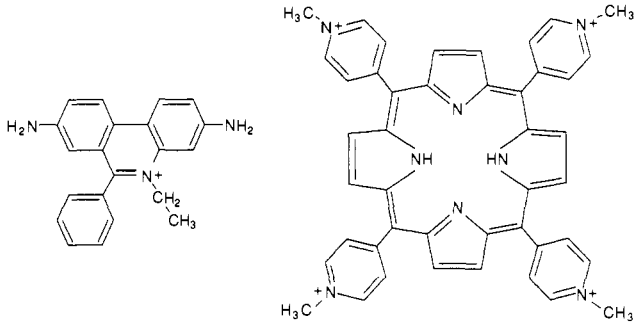

Figure 1. Structures of molecular ions used in this study: (left) ethidium ion, E^+ (right) tetrakis(4-*N*-methylpyridyl)porphine, H_2T4.

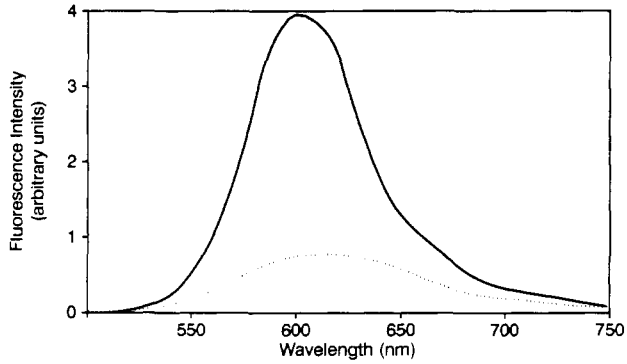

Figure 2. Fluorescence of ethidium ion in the absence (···) and presence (—) of calf thymus DNA.

This study, therefore, permits an analysis of whether the porphyrin-binding mode influences appreciably the efficiency of ethidium fluorescence quenching. Furthermore, the metalloporphyrin derivatives used here were selected so as to make an electron-transfer pathway for quenching less likely than for previously studied systems. Electrochemical investigations of these porphyrin species (H_2T4, NiT4, and ZnT4) have been conducted in water and DMF.[18-22] The reductions of NiT4 and ZnT4 like that for H_2T4 involve the formation of π-radical species; i.e., the metal ions are not readily reduced from the +2 state. All of these porphyrins show quite complicated electrochemical behavior in solution, tending in each case to accept two electrons followed by protonation, disproportionation, or aggregation of the π-radical species thus formed. However, values for the one-electron process $MT4 + e^- \rightarrow MT4^{\bullet-}$ have, with some difficulty, been determined experimentally in water as $E_{1/2} = -0.10$ V_{NHE} and -0.69 V_{NHE} for M = H_2 and Zn, respectively.[19] The reduction potential of NiT4 is estimated to be between those of H_2T4 and ZnT4.[22] This variation in ease of reduction should be reflected in relative efficiencies of quenching, if an electron-transfer pathway is dominant.[6,10] Furthermore, the value for ZnT4 is sufficiently unfavorable that a process in which it accepts an electron from an excited ethidium ion would be expected to be ergonically unfavorable,[6] although some uncertainty exists in as much as none of the potentials were determined in the presence of DNA. The results to be presented below do indeed suggest energy transfer as the primary pathway by which $^*E^+$·DNA is quenched by these porphyrin acceptors and provide evidence for long-range communication of ligands through the double helix.

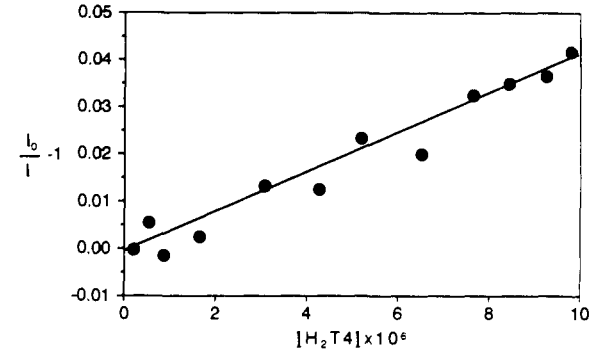

Figure 3. Stern–Volmer plot for the quenching of 5 μM ethidium fluorescence by H_2T4 in the absence of DNA. Solution conditions were 10 mM NaCl; 1 mM phosphate buffer, pH 7.0; and 25 °C.

Experimental Section

Materials and Methods. The DNA employed in these studies was calf thymus type I from Sigma Chemical Co. The nucleic acid was purified by isoamyl alcohol/chloroform extraction using a standard method described previously.[13] Ethidium (E^+) bromide was also supplied by Sigma Chemical Co. and used without further purification. Tetracationic tetrakis(4-*N*-methylpyridyl)porphine, H_2T4, was obtained from Midcentury in the chloride form. The nickel(II) and zinc(II) derivatives of the porphyrin were prepared and purified by literature methods.[23]

Stock solutions of ethidium and porphyrins were used within 1 week of preparation. These stocks were made in 10 mM NaCl and 1 mM phosphate buffer, pH 7.0–7.1. To minimize degradation of the samples, all solutions were kept in the dark until used and stocks were refrigerated. The concentration of stock solutions of DNA (mol of base pairs/L) H_2T4, NiT4, ZnT4, and E^+ were determined spectrophotometrically by using Beer's Law and the following molar absorptivity values, respectively:[21-27] 1.31×10^4 M^{-1} cm^{-1} at 260 nm; 2.26×10^5 M^{-1} cm^{-1} at 422 nm; 1.49×10^5 M^{-1} cm^{-1} at 417.5 nm (20% acetone); 2.04×10^5 M^{-1} cm^{-1} at 437 nm; and 5.86×10^3 M^{-1} cm^{-1} at 480 nm. Absorption spectra were recorded by using a Nicolet 9420 UV/visible spectrophotometer.

Fluorescence spectra were obtained with a SPEX F111 spectrofluorimeter using right-angle geometry. The absorbances of the solutions studied were always less than 0.08 at the excitation (480 nm) and emission (600 nm) wavelengths to minimize both internal shielding and filtering effects (i.e., the so-called "trivial" process in which a photon released by the donor is absorbed by the quencher). All fluorescence experiments were conducted at 25° with acrylic cuvettes.. Emission spectra of blank solutions containing 10 mM NaCl and 1 mM phosphate buffer, pH 7.0–7.1, were subtracted from fluorescence determinations.

Results

(I) Fluorescence Quenching Studies in the Absence of DNA. In the absence of DNA, all the porphyrins studied (H_2T4, NiT4, and ZnT4) are at best weak quenchers of ethidium fluorescence under the conditions of our experiments. At μ = 0.01 M and $[E^+]$ = 5 μM even at 10 μM H_2T4 (for example), less than 10% of the ethidium fluorescence intensity is quenched. The emission spectra obtained in these quenching experiments were reviewed for evidence of enhanced porphyrin fluorescence emission. Not surprisingly, given the marginal degree of quenching and the low quantum yields for fluorescence of these porphyrins in aqueous solution (ϕ = 0.047 for H_2T4 and 0.025 for ZnT4),[28] no such enhanced emissions could be detected within the limits of experimental error. Absorbance measurements failed to reveal any new spectral features and showed only small changes in molar

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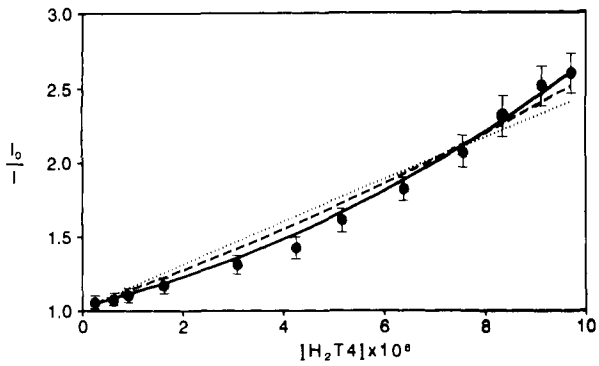

Figure 4. I_0/I for the quenching of 5 μM ethidium fluorescence by H_2T4 in the presence of 200 μM DNA. Various functional forms have been attempted to fit the data. Shown are the "best" fits for linear (···), quadratic (---), and exponential (—) dependencies of I_0/I on quencher concentration.

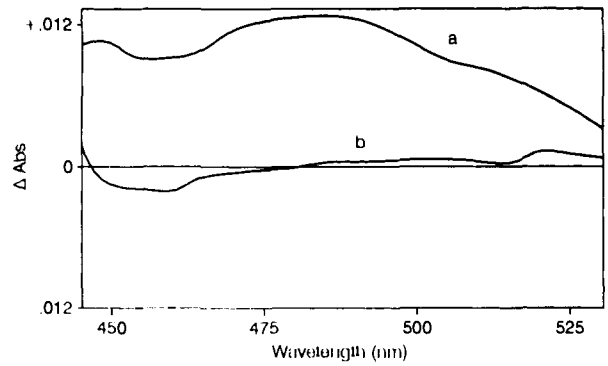

Figure 5. Difference spectra to determine the relative importance of ethidium displacement for the quenching by H_2T4 in the presence of DNA. Details are given in the text.

absorptivity in the range 450–750 nm when ethidium was mixed with each of the porphyrins.

The quenching data are adequately fit by an equation of the form suggested by Stern and Volmer[29,30] Results for H_2T4 as

$$I_0/I = 1 + K_Q[Q] \quad (1)$$

quencher, Q, are shown in Figure 3. For the present experiments, I_0 and I represent, respectively, the ethidium fluorescence intensities in the absence and presence of a porphyrin quencher at a concentration [Q]. Our findings at μ = 0.01 M lead to $K_Q = 4.0 \times 10^3$ M^{-1} for H_2T4 while the value of this constant for NiT4 under the same conditions is 8.3×10^3 M^{-1}. Extensive overlap of the emission bands of ZnT4 (at 626 and 660 nm) with that of E^+ makes analysis of quenching data for this metalloporphyrin at these low efficiencies unreliable.

The ionic strength dependence of K_Q was determined for H_2T4. As might be anticipated for a cationic dye interacting with a cationic quencher, the efficiency of quenching and hence K_Q increases with increasing NaCl concentration. The functional dependence of K_Q on ionic strength is that given by the Debye–Hückel expression:

$$\log K_Q = 3.4 + \frac{2.9\mu^{1/2}}{1 + \mu^{1/2}} \quad (2)$$

(II) Fluorescence Quenching in the Presence of DNA. The binding of ethidium ion to DNA leads to a marked increase in the dye's quantum efficiency for fluorescence as shown in Figure 2. Addition of the cationic, water-soluble porphyrins H_2T4, NiT4, or ZnT4 leads to a quenching of this fluorescence. Figure 4 shows the result of one study obtained at $[E^+] = 5$ μM and [DNA] = 200 μM. Note not only the magnitude of the quenching as compared to that obtained in the absence of DNA as shown in Figure 3, but also that the plot of I_0/I vs $[H_2T4]$ in Figure 4 is not linear. Similar results are obtained for NiT4 and ZnT4.

We have two types of experimental evidence that the quenching observed in these studies is not simply due to the porphyrin-induced displacement of ethidium ion from DNA. First, at some conditions of DNA and porphyrin concentration, the ethidium fluorescence is quenched to an extent appreciably greater than would be obtained if the ion were displaced from the nucleic acid. Second, we have recorded difference spectra that take advantage of the wavelength and molar absorptivity changes in the E^+ spectrum upon complexing with DNA. The sensitivity of the method can be demonstrated by considering the hypothetical case in which half the drug is displaced. A difference spectrum is generated by first adding the spectrum of 2.5 μM E^+ bound to DNA to that of 2.5 μM E^+ free in solution and substracting from the resultant the spectrum of 5 μM E^+ bound to DNA. The resulting difference, reflecting the spectral bathochromic shift and hypochromicity of the ethidium·DNA complex, is shown in Figure 5 as curve a. This then is the difference spectrum expected when half the ethidium is displaced from the nucleic acid. Curve b is generated by first placing in the sample compartment a solution containing 5 μM E^+, DNA, and enough porphyrin to quench 50% of the fluorescence. For 100 μM DNA, for example, 4 μM H_2T4 is required. The reference compartment contains DNA and a porphyrin concentration equal to that in the sample compartment. The resulting *experimental* spectrum is then due to contributions from ethidium at a total concentration of 5 μM when half its fluorescence has been quenched. Next, from this spectrum is subtracted one of 5 μM E^+ totally bound to DNA (no H_2T4 added), giving curve b. As may be seen from Figure 5b, the ethidium spectrum is nearly identical in the presence and absence of H_2T4, indicating at most a very low level ($\leq$10%) of displacement of the dye by H_2T4 even under conditions at which the ethidium fluorescence is quenched by 50%. Taking into account the relative efficiencies of fluorescence emission for bound and free ethidium, 50% quenching would require nearly 60% of the drug be displaced from the nucleic acid if this were the sole mechanism operating. Therefore, we conclude that the primary pathway for porphyrin quenching *does not* involve displacement of ethidium ion.

Table I. Quenching Constants for Region of Influence Model[a] ($[E^+] = 5$ μM, μ = 0.01 M, pH 7, 25 °C)

quencher	[DNA] × 10^4	$\lambda \times 10^{-4}$
H_2TMpyP-4	1.0	20.2 ± 3.3
	2.0	9.6 ± 2.2
	3.0	6.9 ± 2.8
	3.8	5.9 ± 3.1
NiTMpyP-4	1.0	14.2 ± 2.4
	2.0	6.6 ± 2.9
	3.0	4.0 ± 3.6
	4.0	3.1 ± 3.8
ZnTMpyP-4	1.0	15.3 ± 2.2
	2.0	6.4 ± 2.9
	3.0	4.4 ± 3.5
	4.0	3.6 ± 3.9

[a] $I_0/I = e^{\lambda[Q]}$.

Emission spectra of H_2T4 and ZnT4 (NiT4 does not fluoresce appreciably) were compared for these porphyrins when bound to DNA in the presence and absence of ethidium ion. Evidence of *enhanced fluorescence emission* was found for both the nonmetallo and zinc derivatives in the presence of ethidium. The extent of enhancement increases with decreasing DNA concentration and under the conditions of our experiments does not exceed 10–15%.

Returning to the quenching profile of Figure 4, a number of functional forms were considered (linear, quadratic, exponential) to analyze the data.[30] For all twelve cases (three porphyrins, each at four DNA concentrations), the best fit was obtained with an equation of the form $I_0/I = e^{\lambda[Q]}$. Table I provides a listing of the λ values for each of the systems studied. The theoretical basis for this functional form will be considered in the Discussion.

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Finally, the effects of ethidium ion concentration and ionic strength were also considered. At [DNA] = 1×10^{-4} and [E^+] = 1 μM, μ = 0.01, $\lambda = 16.1 \times 10^4$ and μ = 0.11, $\lambda = 15.3 \times 10^4$. Results at μ = 0.60, and μ = 1.5, could be fit by linear plots with $K_Q = 6.1 \times 10^4$ and 7.3×10^4, respectively. Varying [DNA] at [E^+] = 1 μM and μ = 0.11 gives [DNA] = 2×10^{-4}, $\lambda = 7.4 \times 10^4$; [DNA] = 3×10^{-4}, $\lambda = 4.0 \times 10^4$; and [DNA] = 4×10^{-4}, $\lambda = 3.5 \times 10^4$.

Discussion

Using literature values[4,12] for binding constants and exclusion parameters,[31] at μ = 0.01 M we calculate that ≥98.9% of total ethidium and ≥97.8% of the porphyrins are DNA-bound under the conditions of these experiments. As discussed in the Results, both the extent of fluorescence quenching and the difference absorption experiments allow us to conclude that the extent of binding of these drugs is not markedly affected by their concurrent presence, at least at the low drug loads used here. Supporting this conclusion are calculations performed for other systems under similar conditions[6] which show that, in the absence of large cooperativity effects, the binding of one drug does not greatly impact the binding of a second species as long as the drug load is moderate. The analysis of quenching data for these systems is thus simplified by not having to consider a displacement pathway as a major contributor.

The results shown in Figures 3 and 4 are for experiments conducted at the same ethidium concentration (5 μM) over the same concentration range of H_2T4 (≤10 μM) at μ = 0.01 M and T = 25 °C. They differ only in the presence of 200 μM DNA in the experiments of Figure 4. Whereas only 5% of the ethidium fluorescence is quenched at 10 μM H_2T4 in the absence of DNA, greater than 60% quenching is observed with 200 μM DNA *without appreciable ethidium displacement*. As seen in Figure 4, the dependence of I_0/I on [H_2T4] is not linear excluding the possiblity that either a dynamic or static mechanism alone could account for the results.[30] A combined pathway involving both dynamic and static quenching leads to a quadratic dependence for I_0/I vs [H_2T4], but this functional form accounts for neither the profile of Figure 4 nor those obtained at other DNA concentrations or for other porphyrins. In contrast, an exponential dependence like that arising for a "sphere of action" pathway for quenching[30] satisfactorily fits all the data.

The model for the sphere of action quenching mechanism is based upon defining a critical distance, r_0, such that fluorophores within this distance of a quencher at the instant of excitation are totally deactivated. Fluorophores at greater distances are unaffected. The basis for the mathematical model is the calculation of $P(0)$, the probability that no quencher is within a sphere of radius r_0 from the fluorophore as center. Only such "isolated" fluorophores emit photons when excited. A Poisson distribution of molecules has proved satisfactory in calculating $P(0)$ in solution.[30] An analogous approach is considered here to account for the quenching results—that is, a critical distance is defined within which quenching is complete and outside of which quenching is negligible. However, in the present case, the donors and quenchers are bound to a one-dimensional polymer as represented in Figure 6 and the critical distance is given in units of base pairs, σ. We introduce the following *mathematical model*, which assumes (i) no cooperativity and (ii) that an "average" duplex of N base pairs can be used to characterize the system. In other words, the duplexes are considered to be sufficiently long that end effects can be neglected and no other important properties of the system depend on the actual length of a given double helix. Recalling that DNA concentrations are expressed in moles of base pairs per L, we can express the number of ethidium ions per duplex, [e^+], as

$$[e^+] = [E^+]N/[\text{DNA}] \qquad (3)$$

Let l = number of binding sites available per duplex for porphyrins. To calculate this term, we need to know the number of base pairs covered by a drug molecule;[31] for ethidium ion, this value has been determined as 2.[32] Then

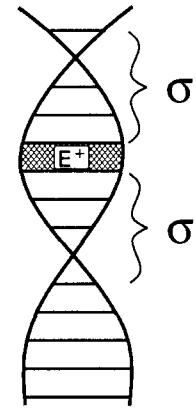

Figure 6. In a region of influence model, porphyrin quenchers within σ base pairs of a bound ethidium ion are assumed to quench the ethidium fluorescence completely.

$$l \approx N\left(1 - \frac{2[E^+]}{[\text{DNA}]}\right) \qquad (4)$$

$P(0)$ is the probability that no porphyrin will be located within σ base pairs of a given E^+ ion (cf. Figure 6). With the assumption that the system shows no cooperativity and under the experimental conditions of low drug load, porphyrins can be treated as if they occupy binding sites at random.[33] Then, for the first porphyrin molecule, the probability that it will not be within σ base pairs on either side of the E^+ shown in Figure 6 is given by $(l - 2\sigma)/l$. The second porphyrin has fewer sites available to it—by using the symbol n for the number of base pairs covered by a porphyrin, the probability that this second porphyrin will not be within σ base pairs of the ethidium ion is $(l - 2\sigma - n)/(l - n)$. For a porphyrin density per duplex equal to $[Q]N/[\text{DNA}]$, we obtain

$$P(0) = \left(\frac{l - 2\sigma}{l}\right)\left(\frac{l - 2\sigma - n}{l - n}\right)\left(\frac{l - 2\sigma - 2n}{l - 2n}\right)\cdots$$

or

$$P(0) = \prod_{i=0}^{\frac{[Q]N}{[\text{DNA}]}-1}\left(\frac{l - 2\sigma - in}{l - in}\right)$$

Taking logarithms, we obtain

$$\ln P(0) = \sum_{i=0}^{\frac{[Q]N}{[\text{DNA}]}-1} \ln\left(1 - \frac{2\sigma}{l - in}\right)$$

In the experiments being analyzed here, in has a *maximum* value[13,15] of about 10% of l and the ct DNA used has not been

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(33) It is our view that ZnT4 and other groove-binding metallodervatives prefer, but are not limited to, regions of DNA rich in AT base pairs (ref 13 and Ward, B.; Skorobogarty, A.; Dabrowiak, J. C. *Biochemistry* **1986**, *25*, 7827). Similarly, intercalation is favored in regions of DNA that contain appreciable numbers of GC sites. However, this does not imply a restriction to regions having sequences of GC base pairs. We have shown, for example, that H_2T4 intercalates into poly(dA-dC)·poly(dT-dG). (Gibbs, E. J.; Maurer, M. C.; Zhang, J. H.; Reiff, W. M.; Hill, D. T.; Malicka-Blaszkiewicz, M.; McKinnie, R. E.; Liu, H.-Q.; Pasternack, R. F. *J. Inorg. Biochem.* **1988**, *32*, 39). Thus, intercalation can occur into GC–AT junctions or other regions of DNA that have sufficient GC content to stabilize the duplex. The precise content and distribution has not as yet been determined. Thus, binding is not totally random but to a first approximation this assumption is probably acceptable and especially under conditions of low drug load.

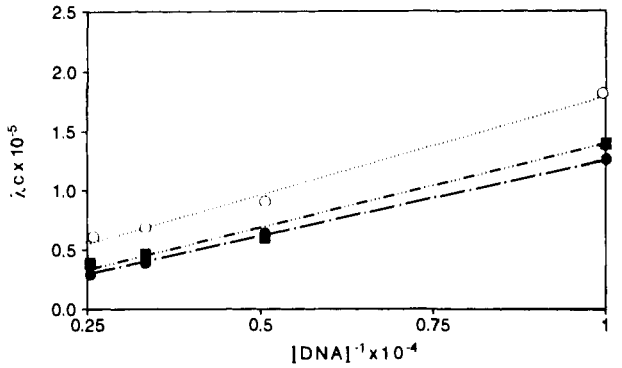

Figure 7. λ_c vs $[DNA]^{-1}$ for H_2T4 (○), ZnT4 (■), and NiT4 (●) at μ = 0.01 M, $[E^+]$ = 5 μM (see Table I).

sonicated and thus consists of long strands. For these experimental conditions, we assume that σ is small compared to $l - in$. Then

$$\ln P(0) = -2\sigma \sum_{i=0}^{\frac{[Q]N}{[DNA]}-1} \frac{1}{l - in}$$

The first several terms of the summation can be written as

$$\ln P(0) \approx -2\sigma\left[\frac{1}{l} + \frac{1}{l-n} + \frac{1}{l-2n} + \ldots\right] \quad (5)$$

but since $in \leq 0.1l$, eq 5 can be approximated as

$$\ln P(0) \approx \frac{-2\sigma}{l}\frac{[Q]N}{[DNA]} \quad (6)$$

Substituting for l as in eq 4 allows this expression to be rewritten as

$$\ln P(0) \approx \frac{-2\sigma[Q]}{\left(1 - \frac{2[E^+]}{[DNA]}\right)[DNA]} \quad (7)$$

Then $P(0) \approx e^{-\lambda[Q]}$ and $I_0/I \approx \exp(\lambda[Q])$ where $\lambda = 2\sigma/(1 - 2[E^+]/[DNA])[DNA]$. (For the systems studied here, $[E^+]/[DNA] \leq 0.05$.) We next define $\lambda_c = \lambda(1 - 2[E^+]/[DNA])$ so that

$$\lambda_c = \frac{2\sigma}{[DNA]} \quad (8)$$

and, therefore, the model predicts that, for experiments conducted at different DNA concentrations, a plot of λ_c vs $[DNA]^{-1}$ should be linear with a slope of 2σ. Such graphs of λ_c vs $[DNA]^{-1}$ constructed with the data of Table I are shown in Figure 7. The plots are linear for all three porphyrins (H_2T4, NiT4, and ZnT4), and the slopes give values of σ = 8.5, 6.5, and 7.0, respectively.

To test the model further, we considered the effect of lowering the ethidium ion concentration from 5 to 1 μM. If the assumptions and approximations of the model are correct, λ_c should be independent of ethidium concentration. For $\mu \approx 0.01$ and $[DNA] = 1 \times 10^{-4}$ (the nucleic acid concentration that provides the most exacting test), we obtain $\lambda_c = 1.8 \times 10^5$ at $[E^+] = 5$ μM and 1.6×10^5 at $[E^+] \approx 1$ μM.

Interpretation. Using 3.4 Å as the distance between base pairs (it is actually somewhat larger in the presence of an intercalator), we conclude that porphyrins 25–30 Å distant are capable of efficient, virtually total quenching of ethidium fluorescence. This result based upon a "region of influence" model in some respects parallels earlier findings of *enhanced quenching efficiency* when donors and acceptors are simultaneously bound to DNA.[6-10] However, unlike previous results, not only is I_0/I not linearly dependent on the concentration of quencher, but an electron-transfer mechanism is highly unlikely for these porphyrin derivatives. The reduction potential for ZnT4 in solution[19] is such as to make an electron-transfer event from an excited ethidium ion unfavorable based upon free energy considerations.[6] Binding to DNA may have some impact on these values; in terms of electrostatic effects, the reduction of the metalloporphyrin would be even more difficult to accomplish although $*E^+$ would be expected to be a better reducing agent. Less ambigious is the relative insensitivity of the quenching efficiencies observed here for the several porphyrins to reduction potential and therefore to the free energy of a redox process. Therefore, we conclude that electron transfer is not a primary pathway unlike the cases in which MV^{2+} or Cu^{2+} serve as acceptors.[6,9]

Rather, we believe that energy transfer of the Förster type, that is, without photon emission, is involved. The degree of transition dipole coupling (which is the basis of Förster energy transfer) reflects the spectral overlap of the donor and acceptor and their relative orientation and, generally, falls off as the sixth power of distance between them. All three porphyrins studied have absorption bands at or near 600 nm, the wavelength of ethidium fluorescence emission. Furthermore, as would be expected for a Förster pathway, both H_2T4·DNA and ZnT4·DNA show enhanced fluorescence emission when E^+ is present. The degree of enhancement is consistent with the extent of quenching observed (and therefore decreases with increasing DNA concentration for a given set of conditions) and with the relatively low efficiency of porphyrin emission.

The marginally greater quenching efficiency of H_2T4 related to NiT4 and ZnT4 may reflect contributions from an alternative (electron-transfer) mechanism or the fact that the metal-free derivative has a richer absorption spectrum than the other porphyrins at wavelengths above 600 nm. The H_2T4·DNA complex has four bands between 500 and 750 nm at 525, 566, 595, and 658 nm. NiT4 like most metalloporphyrins has one major band in this range (at 540 nm) for its DNA complex with a pronounced shoulder at 576 nm. A smaller broad absorption band exists at 632 nm for the NiT4·DNA complex extending to the 576-nm shoulder. The spectrum of the ZnT4·DNA complex, on the other hand, has its main visible (as contrasted to Soret) band at 561 nm and a pronounced shoulder at 601 nm. These spectral positions result in the ZnT4 spectrum showing more extensive overlap than the NiT4 spectrum with the main emission feature of ethidium. On the other hand, both ethidium and NiT4 intercalate into ct DNA whereas ZnT4 binds externally in grooves. The degree of coupling of transition dipoles is influenced by the relative orientation of molecules[34] which is expressed in terms of an orientation factor, K^2. For ZnT4, on the basis of the results of Geacintov et al.,[17] K^2 can be estimated as about 1.8, which has the effect of lowering the efficiency of transfer relative to parallel, aligned transition dipoles as expected for intercalators like NiT4. Thus, that the nickel(II) and zinc(II) derivatives are nearly identical in their quenching properties may reflect two offsetting factors: ZnT4 has a better absorption spectrum for quenching, but NiT4 is better oriented. Still, a main point to be taken from these studies is that the binding mode of the porphyrin appears to play little role in quenching efficiency. We have previously shown that, as the ionic strength of the medium is raised from 0.01 to 0.11 M, H_2T4 changes from being virtually totally intercalated into ct DNA to being equally distributed between intercalated and groove bound.[15] Consistent with the interpretation of the metalloporphyrin results, we obtain nearly identical quenching results for H_2T4 at the two ionic strengths ($\lambda = 1.6 \times 10^5$ at μ = 0.01 and 1.5×10^5 at μ = 0.11). The value of σ = 8.5 obtained at μ = 0.11 M is the same at that derived from the results at μ = 0.01.

The avidity of binding of drugs to nucleic acids decreases with increasing Na^+ concentration in a well-understood manner. Thus, by μ = 1.5 M ethidium and H_2T4 are free in solution even in the presence of 1×10^{-4} M ct DNA. The quenching profile under these conditions is linear, giving a $K_Q = 7.3 \times 10^4$ M^{-1}. Applying eq 2 to these very high salt conditions provides a calculated K_Q

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$= 1.0 \times 10^5\ M^{-1}$, a result in reasonable agreement when one considers the approximate nature of the Debye-Hückel equation under these conditions.

Concentration effects have been shown to play an important part in quenching efficiency when donors and acceptors are simultaneously bound to DNA, as is the case here at low salt concentration.[16] On the other hand, drug mobility on the one-dimensional surface[10] is unlikely to be of significance in these systems since the rate of deintercalation of NiT4 is orders of magnitude slower than rates of migration of ZnT4 along the DNA helix.[14] If mobility were a determining factor, then NiT4 would show much lower quenching efficiencies than ZnT4. Rather, an important aspect of the role played by the DNA becomes apparent from the distance dependence inherent in the region of influence model proposed here to account for the quenching results. For the usual dipole coupling (Förster) mechanism the efficiency of quenching follows an inverse sixth power dependence on distance between donor and quencher. However, a previous study has shown that this predicted dependence is not observed for fluorophores bound to oligonucleotides and that drug-DNA electronic interactions may be important.[35] These same researchers point out that the data of an earlier report[36] indicate that the efficiency of energy transfer from oligonucleotide-bound excited fluorescein to rhodamine is virtually unchanged whether the two dyes are separated by *four or eight base pairs*. Rapid drop-off of efficiency as predicted by the Förster mechanism occurs only at longer distances. Although admittedly in a highly simplified way, the step function of the region of influence model presented here shows a similar distance profile.

The sphere of action model upon which the present approach is based has been applied to a few solution systems as, for example, oxygen quenching of perylene.[30] In general, the sphere radius measured is only slightly larger than the sum of the fluorophore and quencher radii and it is concluded that the two species must be nearly in contact for quenching to occur.[37] This is in marked contrast to the results obtained here. We believe the results of this study point out that, in agreement with the findings of Cooper and Hagerman[35] and Cardullo et al.,[36] an important influence of the DNA matrix is to permit extensive electronic communication between bound drug molecules—an influence that serves to raise the efficiency of transition dipole coupling at long distances. Electronic communication of this type can contribute in part to properties as diverse as the unusual spectral features of DNA-bound chromophores (e.g., large bathochromic shifts are sometimes observed that cannot be simulated with mono- or dinucleotides)[13,15,38] and drug synergism. We are currently proceeding with studies to consider the role base specificity, porphyrin basicity, and metal redox properties play in this communication process.

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Properties of the Excited-Singlet States of Bacteriochlorophyll *a* and Bacteriopheophytin *a* in Polar Solvents

Michael Becker,† V. Nagarajan, and William W. Parson*

Contribution from the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received December 3, 1990. Revised Manuscript Received May 6, 1991*

Abstract: Several approaches were used to characterize the lowest excited-singlet states of bacteriochlorophyll *a* (BChl-*a*) and bacteriopheophytin *a* (BPh-*a*). Fluorescence quantum yields for BChl-*a* measured in five polar solvents ranged from 11% in methanol to 20% in pyridine; BPh-*a* had a fluorescence yield of 8% in methanol and 10% in acetone. In each solvent, the emission spectrum did not show mirror symmetry with the absorption spectrum: the fluorescence spectrum was narrower, and its vibrational shoulder was less pronounced and occurred at shorter wavelengths than predicted from the absorption spectrum. The apparent temperature of the excited state, as calculated by the Stepanov relationship, was higher than the ambient temperature (296 K) by about 30 K. The anomalous fluorescence properties can be explained qualitatively by inhomogeneous broadening and vibronic mixing in the excited state. In spite of these anomalies, the fluorescence yields are close to the yields calculated on the basis of previously measured fluorescence lifetimes (Connolly, J. S.; Samuel, E. B.; Janzen, A. F. *Photochem. Photobiol.* **1982**, *36*, 565-574) and calculated radiative lifetimes, indicating that the overall vibrational partition functions are similar in the ground and excited states. Picosecond transient absorption spectra were measured with BChl-*a* in methanol, 1-propanol, and pyridine, using 605-nm excitation pulses with a width of about 0.8 ps. The spectra in the 780-nm region are characterized by a sharp negative trough that shifts to the red with time. The shifting probably reflects adjustments of the stimulated-emission spectrum in response to dielectric relaxation of the solvent around the excited molecule. The relaxation in pyridine is well described by a single exponential with a time constant of 2.7 ± 0.1 ps. The shifting in the alcohols can be fit by biexponentials with time constants of 1.5 ± 0.2 and 18 ± 3 ps in methanol, and 1.1 ± 0.4 and 82 ± 25 ps in 1-propanol. By using an actinometric method to determine the number of molecules in the excited state, the absorption changes at long delay times were used to calculate the $S_{1\rightarrow n}$ absorption spectrum of the thermally equilibrated excited state. The broad, featureless spectrum has an absorption cross section $\sigma_{1\rightarrow n}$ of $(1.0 \pm 0.2) \times 10^{-16}$ cm^2 at 750 nm.

Bacteriochlorophyll (BChl, Figure 1) is a Mg tetrahydroporphyrin that functions both to capture photons and to transfer electrons in photosynthetic bacteria. Figure 1 shows the absorption spectrum of BChl-*a* in pyridine. The four absorption bands can be described in terms of electronic transitions among the two highest filled molecular orbitals and two normally unoccupied orbitals.[1] The strong absorption band near 770 nm, termed the

†Present address: Strukturforschung, Max-Planck Institut für Biochemie, W-8033, Martinsried bei München, Germany.

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